

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
12 September 2003 (12.09.2003)

PCT

(10) International Publication Number
WO 03/074084 A1

(51) International Patent Classification⁷: **A61K 45/06**,
38/17, A61P 19/08, 19/10

(21) International Application Number: PCT/JP03/02259

(22) International Filing Date: 27 February 2003 (27.02.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2002-055356 1 March 2002 (01.03.2002) JP

(71) Applicant: **SANKYO COMPANY, LIMITED** [JP/JP];
5-1, Nihonbashi Honcho 3-chome, Chuo-ku, Tokyo 103-
8426 (JP).

(72) Inventors: **KUMAKURA, Seiichiro**; c/o SANKYO
COMPANY, LIMITED, 2-58, Hiromachi 1-chome, Shina-
gawa-ku, Tokyo 140-8710 (JP). **NAKAJIMA, Tomoko**;
c/o SANKYO COMPANY, LIMITED, 2-58, Hiromachi
1-chome, Shinagawa-ku, Tokyo 140-8710 (JP).

(74) Agents: **OHNO, Akio** et al.; c/o Sankyo Company, Lim-
ited, 2-58, Hiromachi 1-chome, Shinagawa-ku, Tokyo 140-
8710 (JP).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE,
SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC,
VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI,
SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN,
GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: A PHARMACEUTICAL COMPOSITION

(57) Abstract: A novel pharmaceutical composition containing: (i) a complex comprising at least one substance selected from the group consisting of osteoclastogenesis inhibitory factor (OCIF), analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof; and (ii) a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins, shows prolonged retention of said OCIF, analogue thereof or variant thereof in the bloodstream after administration and enhanced pharmacological activity, making it useful in the treatment and prophylaxis of bone metabolic diseases.

BEST AVAILABLE COPY



WO 03/074084 A1

PHARMACEUTICAL COMPOSITION COMPRISING OSTEOCLASTOGENESIS INHIBITORY FACTOR

Technical field of the invention

The present invention relates to pharmaceutical compositions, kits and agents for the prevention or treatment of bone metabolic diseases which contain: (i) a complex of at least one osteoclastogenesis inhibitory factor (referred to hereinafter as OCIF), an analogue thereof or a variant thereof, and at least one polysaccharide or derivative thereof; and (ii) a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins. The present invention further relates to the use of said compositions, kits and agents in the treatment or prevention of bone metabolic diseases; to a method for the prevention or treatment of bone metabolic diseases which comprises the administration to a patient in need thereof an effective amount of (i) a complex of at least one OCIF, analogue thereof or variant thereof, and at least one polysaccharide or derivative thereof; and (ii) at least one substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins; and to a method for the preparation of the compositions of the present invention.

Background to the Invention

Bones contain about 99% of the total calcium present in the living body, and therefore play an important role not only in supporting the body but also functioning as the largest storage organ of calcium in the body. The bones play an important role in maintaining homeostasis of the calcium. It is known that the activation of osteoclasts, which play an important role in bone resorption, causes excessive flow of calcium into the blood from the bones to break the homeostasis of calcium in the blood, thus inducing hypercalcemia. This induction of hypercalcemia by the activation of osteoclasts and promotion of bone resorption can be caused by cytokines that are secreted abnormally as a result of the spread of cancer to the bone [e.g. see Jean-Jacques Body, Current and Future Directions in Medical Therapy: Hypercalcemia, CANCER Supplement, 88(12), 3054-3058 (2000)]. The prognosis for patients suffering from cancerous hypercalcemia is generally poor and it is

therefore highly desirable to find an effective treatment for this condition.

In rheumatism such as rheumatoid arthritis and the like or osteoarthritis, the abnormal formation or abnormal activation of osteoclasts is known to be one of the main causes of various of the symptoms that present in the bones and joints of patients suffering from these conditions [e.g. see E. Romas, M. T. Gillespie and T. J. Martin, Involvement of Receptor Activator of NF- κ B Ligand and Tumor Necrosis Factor- α in Bone Destruction in Rheumatoid Arthritis, *Bone*, 30(2), 340-346 (2002)]. The pain in the joints and bones due to rheumatism such as rheumatoid arthritis and osteoarthritis is extremely intense and is seriously deleterious to the quality of life of patients suffering from these conditions. Again, it is therefore highly desirable to find an effective treatment for these conditions.

Osteoclasts are also known to play a role in osteoporosis. The balance of bone resorption promoted by osteoclasts and bone formation promoted by osteoblasts gradually inclines towards bone resorption due to the reduced secretion of female hormones after menopause or due to aging, as a result of which the bone mineral density is lowered and osteoporosis is caused if this reduction in bone mineral density is sufficiently severe. When aged patients with a high risk of osteoporosis suffer a fracture, the possibility that they will become bedridden is high, and this has become a social issue as a result of the increasingly aged population in all parts of the world [e.g. see Bruno Fautrel and Francis Guillemin, Cost of illness studies in rheumatic diseases, *Current Opinion in Rheumatology*, 14, 121-126 (2002)]. An effective means of treating and preventing osteoporosis is therefore keenly sought after.

Conventional treatments for these conditions include the supplementation of hormones such as estrogen and the use of agents that suppress the activity of osteoclasts such as bisphosphonates or calcitonins [e.g. see Mohammad M. Iqbal and Tanveer Sobhan, Osteoporosis: A Review, *Missouri Medicine*, 99(1), 19-23 (2002)]. However, hormones can have undesirable side effects such as the raised risk of oncogenesis, the induction of endometriosis and abnormal bleeding from genitals [e.g. see Joyce Penrose White and Judith S. Schilling, Postmenopausal Hormone Replacement: Historical Perspectives and Current Concerns, *Clinical Excellence for Nurse Practitioners*, 4(5), 277-285 (2000)]. Although it is known that

bisphosphonates easily bind excess calcium in the blood and accumulate at bone, some researchers doubt to what extent the strength of bone can be improved thereby. Furthermore, it has also been reported that there is a danger of impaired kidney function associated with their use [e.g. see Jonathan R. Green, Yves Seltenmeyer, Knut A. Jaeggi and Leo Wildler, Renal Tolerability Profile of Novel, Potent Bisphosphonates in Two Short-Term Rat Model, *Pharmacology and Toxicology*, 80, 225-230 (1997)]. As for calcitonin, the increase in bone mineral density obtained with their use is, unfortunately, transient. It has also been reported that interruption of administration of calcitonin can cause a regression of the condition being treated, while the effectiveness of calcitonins originating from animals other than humans can be reduced after prolonged treatment as a result of the appearance of circulating antibodies to the calcitonin in the human body [S. L. Porcel, J. A. Cumplido, B. dela Hoz, M Cuevas and E. Losada, Anaphylaxis to calcitonin, *Allergologia et Immunopathologia*, 28(4), 243-245 (2000)].

As explained above, osteoclasts play a major role in promoting bone resorption which is an important factor governing the increase of calcium concentration in the blood. However, it is believed that none of the above-mentioned existing medicines have any activity in suppressing the formation of osteoclasts. Consequently, none of these conventional medicines is suitable for fundamental treatment of bone metabolic diseases as they are only able to manage the symptoms rather than address the causes.

More recently, OCIF has been demonstrated to be an endogenic protein which inhibits differentiation of an osteoclast precursor cell to an osteoclast and/or the bone resorption activity of the mature osteoclast (see WO-A-96/26217, EP-A-0816380 and US2002/0051969). OCIF is also known as osteoprotegerin (see WO-A-97/23614). In view of the fact that the abovementioned bone metabolic diseases such as hypercalcemia, osteoporosis and rheumatoid arthritis all result at least to some extent from bone resorption, it was hoped that these diseases could be successfully treated using OCIF due to this ability to suppress the formation of the osteoclast itself and/or to suppress the bone resorption activity of the mature osteoclast. However, OCIF is a basic protein which has an isoelectric point of around 9, and it disappears very rapidly from the bloodstream after administration. An attempt to address this

problem is disclosed in WO-A-2000/24416, US 2001/0031725 A1 and EP-A-1127578 where the length of time that OCIF remains in the blood after administration (and hence the effect of the OCIF) was prolonged to a certain extent by co-administering the OCIF with a polysaccharide such as heparin or dextran sulfate. However, the prolongation of the retention time achieved as a result may not be sufficient to give the sort of prolonged retention of OCIF in the blood that would make it a genuine candidate for use in the treatment of bone metabolic diseases such as hypercalcemia, osteoporosis and rheumatism. There is, therefore, a need for an improved means of prolonging the length of time that OCIF is retained in the bloodstream after administration. In EP-A-1270015, which was published after the priority date of the present invention, a complex of OCIF and a polysaccharide such as heparin or dextran sulfate is disclosed with a prolonged retention time in the bloodstream after administration.

Non-steroidal anti-inflammatory drugs (referred to hereinafter as NSAIDs) have been widely used for clinical purposes for the treatment of inflammatory diseases such as pyrexia, pain and edema. NSAIDs act by inhibiting the synthesis of prostaglandins; it is believed that they inhibit the action of prostaglandin cyclooxygenase (COX) which is an enzyme active in a crucial step in the production of prostaglandins from arachidonic acid. There are two isozymes, called COX-1 and COX-2, present in COX. COX-1 is normally present in the stomach, the intestines, the kidneys and other tissues and serves to produce prostaglandin which functions physiologically, while COX-2 is induced by inflammatory cytokines and endotoxins, such as IL-1, TNF α and the like, and is expressed specifically at an inflammatory site to produce prostaglandin which functions as a mediator of inflammatory reactions. Although there has been some suggestion that NSAIDs such as COX-2 inhibitors may have some activity in preventing bone resorption (see, for example, US 5,908,858), this only appears to be achievable with high doses of said NSAIDs, these doses being higher than those required to treat or prevent inflammation.

Recently, in WO-A-2001/03719 it has been suggested that OCIF can be used in combination with various substances that stimulate bone formation. A long list of substances that might be suitable bone formation stimulants is provided, one such possibility being NSAIDs. No actual examples of a combination of OCIF and a

NSAID are disclosed.

It will be understood from the review above that there is, therefore, a need for an improved means of prolonging the length of time that OCIF is retained in the bloodstream after administration. Furthermore, there is a need to enhance the pharmacological activity of the OCIF. The inventors of the present invention have performed extensive research to try to achieve these goals. They have found that a complex of OCIF and a polysaccharide has a significantly improved retention in the bloodstream as compared with conventionally known compositions of OCIF and polysaccharide, and further found that the pharmacological effect can be reinforced by combined use of the complex and a NSAID.

Brief Summary of the Invention

It is therefore an object of the present invention to provide a preparation comprising OCIF which enables the length of time that OCIF is retained in the bloodstream after administration to be prolonged while also enhancing the pharmacological activity of said OCIF, thus providing an agent in which the effect of OCIF in the treatment and prophylaxis of bone metabolic diseases which are mediated by osteoclasts, such as hypercalcemia, osteoporosis and rheumatism, is enhanced and prolonged.

Other objects and advantages of the present invention will become apparent as the description proceeds.

Thus, in a first aspect of the present invention there is provided a pharmaceutical composition which contains:

- (i) a complex comprising at least one substance selected from the group consisting of osteoclastogenesis inhibitory factor (OCIF), analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof; and
- (ii) a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins.

In a second aspect of the present invention, there is provided an agent for the prevention or treatment of bone metabolic diseases which contains as active ingredients:

- (i) a complex comprising at least one substance selected from the group consisting of osteoclastogenesis inhibitory factor (OCIF), analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof; and
- (ii) a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins.

In a third aspect of the present invention, there is provided a kit including a plurality of containers in which at least one of said containers contains a complex comprising at least one substance selected from the group consisting of osteoclastogenesis inhibitory factor (OCIF), analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof, and at least one different container contains a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins.

In a fourth aspect of the present invention, there is provided a method for the prevention or treatment of bone metabolic diseases in a mammal, which may be human, comprising administering to a patient in need thereof effective amounts of the following active ingredients:

- (i) a complex comprising at least one substance selected from the group consisting of osteoclastogenesis inhibitory factor (OCIF), analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof; and
- (ii) a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins.

In a fifth aspect of the present invention, there is provided the use of a complex comprising at least one substance selected from the group consisting of osteoclastogenesis inhibitory factor (OCIF), analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of

polysaccharides and derivatives thereof in the manufacture of a medicament for use with a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins for the prevention or treatment of bone metabolic diseases.

In a sixth aspect of the present invention, there is provided the use of a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins in the manufacture of a medicament for use with a complex comprising at least one substance selected from the group consisting of osteoclastogenesis inhibitory factor (OCIF), analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives for the prevention or treatment of bone metabolic diseases.

Preferred pharmaceutical compositions, agents for the prevention or treatment of bone metabolic diseases, kits, methods for the prevention or treatment of bone metabolic diseases and uses of said complex and said substances that suppress the production of prostaglandins and/or compete with a biological action of prostaglandins in the manufacture of a medicament for the prevention or treatment of bone metabolic diseases include those wherein:

- (2) the polysaccharide or a derivative thereof is dextran sulfate;
- (3) the polysaccharide or a derivative thereof is dextran sulfate having an average molecular weight of 1800 to 6000;
- (4) the substance that suppresses the production of prostaglandins and/or the substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID);
- (5) the substance that suppresses the production of prostaglandins and/or the substance that competes with a biological action of prostaglandins is a NSAID having cyclooxygenase inhibitory activity;

- (6) the substance that suppresses the production of prostaglandins and/or the substance that competes with a biological action of prostaglandins is a NSAID selected from loxoprofen sodium and celecoxib;
- (7) the substance that suppresses the production of prostaglandins and/or the substance that competes with a biological action of prostaglandins is present in an amount that is sufficient to prevent or treat inflammation when administered alone to the patient but is insufficient to prevent or treat bone metabolic diseases when administered alone to said patient;
- (8) the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said substance selected from the group consisting of polysaccharides and derivatives thereof in said complex is from 1:1 to 1:8;
- (9) the complex comprising at least one substance selected from the group consisting of OCIF, analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof is produced by:
- (a) incubating said at least one substance selected from the group consisting of OCIF, analogues thereof and variants thereof and said least one substance selected from the group consisting of polysaccharides and derivatives thereof under alkaline conditions; and
- (b) then removing any free polysaccharides or derivatives thereof not bound to said OCIF, analogues thereof or variants thereof;
- (10) the incubation step (a) in (9) above takes place at a pH of from 9.5 to 12;
- (11) the incubation step (a) in (9) above is performed at a pH of from 10 to 11;
- (12) the removal of free polysaccharides or derivatives thereof in step (b) of (9) to (11) above is effected by gel filtration.

Detailed Description of the Invention

We have found that by incubating at least one substance selected from OCIF, analogues and variants thereof with at least one substance selected from polysaccharides and derivatives thereof under conditions that result in the formation of a complex in which said one or more substances selected from OCIF, analogues and variants thereof are bound to said at least one substance selected from polysaccharides and derivatives thereof, an agent is thereby produced in which said OCIF or analogue or variant thereof is retained in the bloodstream after administration thereof for a prolonged period when compared to the prior art combinations of OCIF and polysaccharides disclosed in WO-A-2000/24416 and EP-A-1127578. By using said complex in combination with a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins, we have found that the pharmacological activity of said OCIF, analogues and variants thereof is significantly and surprisingly enhanced. Thus, by complexing the OCIF or analogue or variant thereof with a polysaccharide or a derivative thereof and using the resulting complex in combination with a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins, the effect of said OCIF or analogue or variant thereof in the treatment and prophylaxis of bone metabolic diseases which are mediated by osteoclasts, such as hypercalcemia, osteoporosis and rheumatism, is prolonged and enhanced.

As noted above, the complexes of the present invention comprise at least one substance selected from OCIF, analogues and variants thereof which are bound to at least one substance selected from polysaccharides and derivatives thereof. In said complex, the OCIF and polysaccharide are bound to each other by a chemical bond such as a covalent bond (e.g. Schiff base formation), an ionic bond or a coordinate bond, or by a non-chemical bond such as a hydrophobic interaction, a hydrogen bond, an electrostatic interaction or affinity binding.

OCIF, an analogue thereof or a variant thereof used in the present invention can be natural type or it can be recombinant type and its origin is not particularly limited. Natural type OCIF means OCIF that is obtained as a naturally produced protein by extraction, purification and/or isolation from an organ, a body fluid, a cell

culture, or a culture medium derived from a human or a non-human animal. Recombinant type OCIF, an analogue thereof or a variant thereof is a recombinant protein obtained by extraction, purification and/or isolation of said protein from a host conventionally used in such techniques such as a prokaryotic host cell (e.g. *Escherichia coli*) or a eukaryotic cell such as a human or a non-human cell line which has been transformed with a vector comprising a polynucleotide which encodes an OCIF, an analogue thereof or a variant thereof [e.g. see the recombinant methods disclosed in EP-A-0816380 (WO-A-96/26217) and WO-A-97/23614].

The origin of the OCIF, analogues thereof and variants thereof used in the present invention is not particularly limited and they can be derived from a human or a non-human animal. Preferably, they can be derived from a mammal such as a human, rat, mouse, rabbit, dog, cat, cow, swine, sheep or goat; or an avian such as a fowl, goose, chicken or turkey. More preferably, they are derived from mammals and most preferably they are derived from a human.

The OCIF or analogue thereof used in the present invention can be a monomer-type OCIF (e.g. in humans a monomer having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000) or a dimer type (e.g. in humans a dimer having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions) [see EP-A-0816380 (WO-A-96/26217)].

In the present invention, an OCIF analogue means a protein encoded by a polynucleotide which exists naturally in the cells, body fluid, and/or organs of a human or non-human animal such as those exemplified above. Specific preferred examples of such analogues include OCIF2, OCIF3, OCIF4 and OCIF5 [see EP-A-0816380 (WO-A-96/26217)]. Such OCIF analogues or active fragments thereof can be obtained by a method such as the following: RNA is extracted from a cell, organ, tissue or body fluid of a human or non-human animal; a first strand of cDNA which is complementary to said RNA is synthesized using a reverse transcriptase, and then a second strand of said cDNA is synthesized using the first as a template and a DNA polymerase; the double-stranded cDNA thus-obtained is inserted into a suitable, conventionally-used expression vector; a suitable, conventionally-used host cell is

then transformed by the vector thus-obtained; the host producing the desired peptide is then screened for using a hybridization technique such as plaque hybridization or phage hybridization using OCIF cDNA or a fragment thereof as a probe under stringent conditions [see EP-A-0816380 (WO-A-96/26217)]; and then finally the desired OCIF analogue is expressed by a conventional technique by the thus-obtained host cell.

In the present invention, an OCIF variant means a protein which has an amino acid sequence wherein one or more than one amino acid residues have been substituted in, deleted from, added to or inserted in the amino acid sequence of an OCIF or an analogue thereof, and still has at least some OCIF activity. Such OCIF variants can be obtained by, for example, the following method: substituting, deleting, adding and/or inserting one nucleotide or more than one nucleotides in a nucleotide sequence encoding OCIF or an analogue thereof using a polymerase chain reaction method (referred to hereinafter as PCR), a genetic recombination method or a nuclease digestion method using an exonuclease or endonuclease such as a restriction enzyme; transforming a eukaryotic host cell such as an animal cell or a prokaryotic host cell such as *Escherichia coli* with an expression vector wherein the obtained nucleotide encoding the desired OCIF variant has been inserted; and then extracting, purifying and/or isolating the desired peptide from the protein-containing fraction produced by a cell culture of said transformed host according to a method well-known to the person skilled in the art.

It is known that OCIF is translated in cells as a polypeptide containing a signal peptide at the amino terminus thereof and that it is then matured by processing involving the removal of said signal peptide [e.g. see the recombinant methods disclosed in EP-A-0816380 (WO-A-96/26217) and WO-A-97/23614]. The OCIF, analogue thereof or variant thereof used in the present invention includes both the polypeptide containing a signal peptide and the matured form thereof. Preferred examples include the OCIF with the signal peptide having amino acids -21 to +380 of SEQ. ID. NO.1 of the sequence listing and the mature OCIF without the signal peptide having amino acids +1 to +380 of SEQ. ID. NO.1 of the sequence listing. Of these, the mature OCIF is particularly preferred.

It is also known that methionine can be added to such a matured form of OCIF, an analogue thereof or a variant thereof, when it is expressed as a recombinant protein in a host cell, especially in a prokaryotic host cell such as *Escherichia coli*. This is achieved by adding a nucleotide triplet having the sequence ATG (AUG) to the 5'-end of a polynucleotide encoding a matured form of OCIF, an analogue thereof or a variant thereof, and inserting the resultant polynucleotide into a suitable expression vector. The desired matured protein having methionine at the amino terminus thereof can be then expressed by a suitable host cell which has been transformed by said recombinant expression vector. Additionally, one or more than one amino acid can be added to said protein at a position next to the amino terminal methionine by the addition of further nucleotide triplets next to the ATG triplet added at the 5'-end of the polynucleotide encoding a matured form of OCIF, an analog thereof or a variant thereof. One or more amino acids can be further added/inserted between the amino terminal methionine and the amino acid +1 of SEQ ID NO. 1 of the sequence listing.

Truncated forms of OCIF wherein a considerable part of the amino acid sequence has been deleted from the carboxy terminus of an OCIF polypeptide are also known to keep at least some OCIF activity [e.g. see EP-A-0816380 (WO-A-96/26217), US2002/0051969 and WO-A-97/23614]. Such truncated types of OCIF retaining at least some of the activity of the full length OCIF polypeptide are also included in the OCIF variants that can be used in the present invention.

Furthermore, OCIF or a truncated form thereof that is fused with the an immunoglobulin domain such as the Fc domain (e.g. a fusion polypeptide in which the Fc domain of human IgG is attached to the carboxy terminus of OCIF) and which retains at least some of the activity of the full length OCIF polypeptide is known (see WO-A-97/23614 and US 6,369,027), and such fusion proteins are also included in the OCIF variants used in the present invention.

It has also been shown that OCIF or an analogue thereof or a variant thereof can be chemically modified and still retain useful activity and, in some cases, may show advantages such as increased stability or decreased immunogenicity. Such chemical modification can involve derivatization at just a single site in the molecule of the OCIF or an analogue thereof or a variant thereof or at more than one site. For

example, it has been shown that OCIF and variants (derivatives) thereof such as a truncated form can be chemically modified with one or more water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose and polyvinylalcohol, and can show improved biological activity as a result (e.g. see WO-A-97/23614 and US 6,369,027). Such chemically modified types of OCIF or an analogue thereof or a variant thereof are also included in the OCIF variants employed in the present invention.

Examples of known OCIF variants that are suitable for use in preparation of the complexes employed in the present invention may include: OCIF-C19S, OCIF-C20S, OCIF-C21S, OCIF-C22S, OCIF-C23S, OCIF-DCR1, OCIF-DCR2, OCIF-DCR3, OCIF-DCR4, OCIF-DDD1, OCIF-DDD2, OCIF-CL, OCIF-CC, OCIF-CDD2, OCIF-CDD1, OCIF-CCR4, OCIF-CCR3, OCIF-CBst, OCIF-CSph, OCIF-CBsp, OCIF-CPst (see WO-A-96/26217, EP-A-0816380 or US2002/0051969), muOPG[22-401]-Fc, muOPG[22-194]-Fc, muOPG[22-185]-Fc, muOPG[22-180]-Fc, muOPG[22-401], muOPG[22-401]C195, muOPG[22-401]C202, muOPG[22-401]C277, muOPG[22-401]C319, muOPG[22-401]C400, muOPG[22-185], muOPG[22-194], muOPG[22-200], muOPG[22-212], muOPG[22-293], muOPG[22-355], huOPG[22-401]-Fc, huOPG[22-201]-Fc, huOPG[22-401]-Fc P26A, huOPG[22-401]-Fc Y28F, huOPG[22-401], huOPG[27-401]-Fc, huOPG[29-401]-Fc, huOPG[32-401]-Fc, MuOPG met[22-194], MuOPG met[22-194] 5k PEG, MuOPG met[22-194] 20k PEG, HuOPG met[22-194]P25A, HuOPG met[22-194]P25A 5k PEG, HuOPG met[22-194]P25A 20k PEG, HuOPG met[22-194]P25A 31k PEG, HuOPG met[22-194]P25A 57k PEG, HuOPG met[22-194]P25A 12k PEG, HuOPG met[22-194]P25A 20k branched PEG, HuOPG met[22-194]P25A 8k PEG dimer, HuOPG met[22-194] P25A disulfide crosslinked (see WO-A-97/23614 or US 6,369,027), OPG[22-194]-Fc, OPG[22-201]-Fc, OPG[22-194]-Fc Δ C, OPG[22-201]-Fc Δ C, OPG[22-194]-FcG₁₀, metFc Δ C-OPG[22-194] (WO-A-2001/17543), OPG[22-194]-Fc Δ C, OPG[22-194]-FcG₁₀, Fc Δ C-OPG[22-194], metFc Δ C-OPG[22-194], metFc Δ C-22-194, OPG[22-194]-Fc, OPG[22-194]-Fc Δ C, metOPG[22-194], metOPG[22-201], OPG[22-293], OPG[22-401] and metFc Δ C-22-194 (WO-A-2001/18203).

Of these, preferred examples include: OCIF-C19S, OCIF-C20S, OCIF-C21S,

OCIF-C22S, OCIF-C23S, OCIF-DCR1, OCIF-DCR2, OCIF-DCR3, OCIF-DCR4, OCIF-DDD1, OCIF-DDD2, OCIF-CL, OCIF-CC, OCIF-CDD2, OCIF-CDD1, OCIF-CCR4, OCIF-CCR3, OCIF-CBst, OCIF-CSph, OCIF-CBsp, OCIF-CPst, muOPG[22-401]-Fc, muOPG[22-194]-Fc, muOPG[22-185]-Fc, muOPG[22-180]-Fc, muOPG[22-401]C195, muOPG[22-401]C202, muOPG[22-401]C319, muOPG[22-401]C400, muOPG[22-194], muOPG[22-200], muOPG[22-212], muOPG[22-293], muOPG[22-355], huOPG[22-401]-Fc, huOPG[22-201]-Fc, huOPG[22-401]-Fc P26A, huOPG[22-401]-Fc Y28F, huOPG[22-401], huOPG[27-401]-Fc, huOPG[29-401]-Fc, huOPG[32-401]-Fc, MuOPG met[22-194]5k PEG, MuOPG met[22-194]20k PEG, HuOPG met[22-194]P25A 5k PEG, HuOPG met[22-194]P25A 20k PEG, HuOPG met[22-194]P25A 31k PEG, HuOPG met[22-194]P25A 57k PEG, HuOPG met[22-194]P25A 12k PEG, HuOPG met[22-194]P25A 20k branched PEG, HuOPG met[22-194]P25A 8k PEG dimer, a HuOPG met[22-194]P25A disulfide crosslinked, OPG[22-194]-Fc, OPG[22-201]-Fc, OPG[22-194]-Fc Δ C, OPG[22-201]-Fc Δ C, OPG[22-194]-FcG₁₀, metFc Δ C-OPG[22-194], OPG[22-194]-Fc Δ C, OPG[22-194]-FcG₁₀, Fc Δ C-OPG[22-194], metFc Δ C-OPG[22-194], metFc Δ C-22-194, OPG[22-194]-Fc, OPG[22-194]-Fc Δ C, metOPG[22-194], metOPG[22-201], OPG[22-293], OPG[22-401] and metFc Δ C-22-194.

OCIF or an analogue or variant thereof employed in the present invention can contain a sugar chain as part of the molecule. Any naturally-produced OCIF or an analogue thereof or recombinant OCIF or analogue or variant thereof can contain a sugar chain which is attached to the OCIF or analogue or variant thereof post-translationally. Naturally-produced OCIF or an analogue thereof containing a sugar chain can be obtained from cell cultures, tissues, organs, body fluids or cell lines derived from human or non-human animals using conventional techniques. Recombinant OCIF or an analogue or variant thereof containing a sugar chain can be obtained from a culture of a eukaryotic host cell transformed using a vector comprising a nucleotide sequence encoding any OCIF or an analogue or variant thereof such as those described and exemplified above. Examples of suitable host cells that can be used which are capable of the post-translational modification of OCIF or an analogue or variant thereof so as to attach a sugar chain include chinese hamster ovary cells (CHO cells) and COS cells [Yasuda, H. et al, Endocrinology, 139,

1329-1337 (1998)]. OCIF or an analogue or variant thereof containing such a sugar chain is suitable for use in the formation of the complexes of the present invention.

If, on the other hand, it is desired to produce a recombinant OCIF or an analogue or variant thereof that does not have a sugar chain that has been added as a post-translational modification, then the preferred host cells are prokaryotic cells such as *Escherichia coli*.

The polysaccharide used in the formation of the complexes employed in the present invention is a polymer (glycan) produced by the glycosidic linkage of two or more monosaccharides, and is preferably a heteropolysaccharide (heteroglycan) consisting of at least two different kinds of monosaccharide. Any polysaccharide, whether naturally-occurring or synthetic can potentially be used in the complex of the present invention.

In the present invention, a derivative of a polysaccharide is a polysaccharide wherein at least a part of said polysaccharide molecule is substituted by one or more than one molecules and/or residues other than a saccharide or sugar. Preferred derivatives include acid esters of polysaccharides, and particularly preferred are sulfate esters of polysaccharides.

Examples of natural polysaccharides suitable for use in the formation of the complexes of the present invention include hyaluronic acid, chondroitin sulfuric acid, dermatan acid, heparan acid, keratan acid, carrageenan, pectin and heparin. Examples of synthetic polysaccharides suitable for use in the formation of the complexes of the present invention include dextran while examples of suitable synthetic polysaccharide derivatives include dextran sulfate. Of the polysaccharides and derivatives thereof, the most preferred for use in the formation of the complexes of the present invention is dextran sulfate.

In the present invention, polysaccharides and derivatives thereof such as dextran sulfate include salts thereof. The most preferred salt of dextran sulfate is the sodium salt thereof. Examples of sodium salts of dextran sulfate include dextran sulfate sodium salt sulfur 5 (referred to hereinafter as DS5; manufactured by Meito

Sangyo Co., Ltd.), and dextran sulfate sodium salt 5000 and dextran sulfate sodium salt 10000 (both of them are manufactured by Wako Pure Chemical Industries, Ltd.).

The molecular weight of a dextran sulfate is calculated as follows.

1) Calculation of the molecular weight of dextran

The molecular weight of dextran can be calculated according to Sato's formulation shown below [e.g. see Manual for Pharmacopoeia of Japan, the thirteenth revision, published by Hirokawashoten (1998), the entry concerning dextran 40] based on the measurement of the limiting viscosity of said dextran. The limiting viscosity can be measured by any suitable method [e.g. see the method disclosed in Manual for Pharmacopoeia of Japan, the thirteenth revision, published by Hirokawashoten (1998), the entry concerning dextran 40].

$$\text{Limiting viscosity} = 9.00 \times 10^{-4} \times \text{molecular weight}^{0.50}$$

2) Measurement of sulfur content

The sulfur content in the dextran sulfate of interest can be measured as a weight % by any conventional technique known in the art, e.g. the method described in the entry concerning dextran sulfate sodium salt sulfur 5 in Pharmacopoeia of Japan [14th revision, published by Jihou (2001)].

While the molecular weight of glucose, which is a unit of dextran, is 180, the actual molecular weight of the glucose unit in a dextran molecule is 162, this value being obtain by subtracting the molecular weight of water from 180 because adjacent glucose units are bound to each other by an α -1,6 glycosidic linkage in the dextran molecule. A hydrogen atom is replaced by a sodium sulfate group (SO_3Na : one gram equivalent = 103) in each glucose unit of a dextran sulfate that is substituted in this manner. Using this information, the degree of substitution of a dextran sulfate molecule (hereinafter referred to as the "substitution degree") can be determined from the following formula:

$$\text{Sulfur content (weight \%)} = [32 \times \text{substitution degree} / (162 + 102 \times \text{substitution degree})] \times 100$$

3) Calculation of the molecular weight of a dextran sulfate

Since, as noted above, the actual molecular weight of the glucose unit in the dextran chain is 162, the molecular weight of a dextran sulfate can be calculated from this information and the degree of substitution determined in (2) above using the following formula:

$$\text{Molecular weight of dextran sulfate} = \text{molecular weight of dextran} \times (162 + 102 \times \text{substitution degree}) / 162$$

It is known that polysaccharides display a distribution of molecular weights, e.g. each different type of dextran sulfate displays a certain molecular weight distribution. The molecular weight of any polysaccharide used in formation of the complexes of the present invention is given as an average molecular weight. The average molecular weight of the polysaccharides used in the present invention is not limited in any way. The range of the average molecular weight of the most preferred polysaccharide derivative of the present invention, dextran sulfate is generally 1500 to 12000, and is more preferably 1800 to 6000. The molecular weight (average \pm standard deviation) of DS5 is about 1950 ± 70 ($n = 7$). The sulfur substitution degree (average \pm standard deviation) of DS5, calculated as described above, is about 0.32 ± 0.01 ($n = 7$). The average molecular weight of dextran sulfate sodium salt 5000 and dextran sulfate sodium salt 10000 are about 5000 and about 10,000, respectively.

The polysaccharides used in preparation of the complexes of the present invention may be used without or with any further purification and/or fractionation therefrom before use. In the present invention, polysaccharides or derivatives thereof do not include any sugar chain which is attached to recombinant OCIF or analogues or variants thereof or to naturally-produced OCIF or analogues or variants thereof post-translationally and/or endogenously in cells or tissues or bodies of human or non-human animals.

The molecular ratio of the substance selected from the group consisting of OCIF, analogues thereof and variants thereof to the substance selected from the group consisting of polysaccharides and derivatives thereof in the complexes of the present

invention will vary depending upon various factors including the identity of the components of said complex and the conditions under which the complex is prepared. There is no particular limitation on the molecular ratio of the substance selected from the group consisting of OCIF, analogues thereof and variants thereof to the substance selected from the group consisting of polysaccharides and derivatives thereof in the complexes of the present invention. In the preferred complexes for use in the present invention comprising a substance selected from the group consisting of OCIF, analogues thereof and variants thereof and a substance selected from the group consisting of polysaccharides and derivatives thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof : said substance selected from the group consisting of polysaccharides and derivatives thereof is from 1:1 to 1:10; more preferably the molecular ratio is from 1:1 to 1:8; and most preferably the molecular ratio is from 1:1 to 1:6.

As has already been mentioned above, OCIF or an analogue or variant thereof can exist as a monomer or can form dimers, such that OCIF or an analogue or variant thereof present in the complexes of the present invention can be a homodimer or a heterodimer, or it can be a homooligomer, heterooligomer, homopolymer or heteropolymer comprising more than two monomeric units of OCIF, an analogue thereof or a variant thereof (e.g. see WO-A-96/26217, US 6,369,027 and US2002/0051969). The molecular ratio of the substance selected from the group consisting of OCIF, analogues thereof and variants thereof to the substance selected from the group consisting of polysaccharides and derivatives thereof in a complex comprising OCIF, or an analogue or variant thereof and polysaccharides or a derivative thereof for use in the present invention is calculated as the number of molecules of polysaccharide or derivative thereof per monomeric unit of OCIF, variant thereof or analogue thereof.

The number of molecules of polysaccharide or derivative thereof in a complex of the present invention can preferably be determined as follows. The neutral sugar content of the tested complex [designated as (x)] and that of a reference sample that contains only the uncomplexed, free OCIF or analogue or variant thereof [designated as (y)] are quantified using the phenol sulfuric acid method (which is described in detail elsewhere in the present application). The amount of

polysaccharide or derivative thereof which is bound to OCIF or an analogue or variant thereof in the tested complex is then determined by subtracting (y) from (x). Using the figure thus obtained, the number of molecules of polysaccharide or derivative thereof which are bound to OCIF or an analogue or variant thereof is calculated according to (I) or (II) below:

(I) The obtained figure for the amount of polysaccharide or derivative thereof which is bound to OCIF or an analogue or variant thereof is divided by the average molecular weight of said polysaccharide or derivative thereof. The resultant figure represents the total number of molecules of polysaccharide or derivative thereof in the test complex.

(II) The obtained figure for the amount of polysaccharide or derivative thereof which is bound to OCIF or an analogue or variant thereof is divided by the amount (mg) of said OCIF, analogue or variant thereof in said complex. The resulting figure, which is the amount of polysaccharide or derivative thereof per 1 mg of OCIF, analogue or variant thereof in the complex, is then used to calculate the number of molecules of polysaccharide or derivative thereof per one molecule of OCIF, analogue or variant thereof on the basis of the average molecular weight of said polysaccharide or derivative thereof and the molecular weight of said OCIF, analogue or variant thereof [e.g. according to Preparation Example 4(d) below].

The number of molecules of OCIF or an analogue or variant thereof in a complex employed in the present invention can preferably be determined using an immunological assay technique, such as those described elsewhere in the present application.

A preferred feature of the complexes used in the present invention that can be used to characterise them is their affinity to heparin. Heparin is a polysaccharide comprising D-glucosamine, D-glucuronic acid and D-iduronic acid which is partially or fully derivatised with sulfate and acetyl groups. A preferred feature of the complexes employed in the present invention is that the strength of adsorption of said complex of OCIF or an analogue or variant thereof to heparin is lower than the strength of adsorption of the free, non-complexed OCIF or analogue or variant thereof. The degree of adsorption can be determined using a column packed with highly cross-linked agarose beads on which has been immobilized heparin (e.g. heparin obtained

from bovine intestinal mucosa). Suitable columns of this type include HiTrap heparin HP column, HiPrep 16/10 Heparin and Heparin Sepharose (all obtainable from Amersham Pharmacia). The strength of adsorption (the affinity) of the complex can be determined according to any suitable method that is well known to the person skilled in the art for determining the affinity of proteins to polysaccharides. Preferably, the degree of adsorption can be determined by comparing the amount of the complex that binds to the heparin column under low ionic strength conditions but that is eluted from said column under high ionic strength conditions with the amount of complex that does not bind to the heparin column under low ionic strength conditions (the ionic strength can be adjusted using the salt of a strong acid such as sodium chloride). Thus, typically the degree of adsorption of the complex to heparin can be determined as follows:

- (a) A column packed with a support such as cross-linked agarose beads on which has been immobilized heparin is equilibrated with a buffer having a relatively low ionic strength (e.g. sodium phosphate buffer containing 0.1-0.8 M sodium chloride).
- (b) The complex of the present invention that is being tested is dissolved in the same low ionic strength buffer as used in (a) and applied to the column and a first eluate is then collected (fraction A).
- (c) The column is then washed further with the same low ionic strength buffer as used in step (a) and a second eluate is collected (fraction B).
- (d) The column is then washed with a buffer having a relatively high ionic strength (e.g. sodium phosphate buffer containing 1.0-2.0 M sodium chloride) and a third eluate is then collected (fraction C).
- (e) The amount of the complex present in each of fractions A, B and C [designated (α), (β) and (γ) respectively] is then determined (e.g. by an immunoassay).
- (f) The degree of adsorption of the complex to heparin is then determined according to the following formula:

$$\text{degree of adsorption} = \frac{(\gamma)}{(\alpha) + (\beta) + (\gamma)}$$

The greater the strength of the binding of the complex to the column, the higher is the value of (γ) (as it can only be removed from the column using eluants having a relatively high ionic strength) and hence the higher is the degree of

adsorption. The degree of adsorption of the complexes of the present invention as measured by the above formula will vary to some extent depending upon the type of heparin column and the conditions under which the determination is carried out. However, the degree of adsorption of free, uncomplexed OCIF is always around 1.0 whereas the degree of adsorption of the complexes of OCIF employed in the present invention is less than 1.0, thus demonstrating that the strength of binding of the complexes comprising OCIF or an analogue or variant thereof used in the present invention to heparin is weaker than the strength of binding of the free, uncomplexed OCIF or analogue or variant thereof (e.g. using porcine heparin immobilized on agarose beads, such as a HiTrap heparin HP column, first and second elutions with 10 mM sodium phosphate buffer containing 0.7 M sodium chloride and a third elution with 10 mM sodium phosphate buffer containing 2.0 M sodium chloride, the degree of adsorption of complexes that are used in the present invention comprising OCIF or a variant thereof or an analogue thereof is not greater than 0.7, preferably not greater than 0.6 and particularly preferably not greater than 0.5).

Another preferred feature of the complexes that are employed in the present invention that can be used to characterise them is the ratio of the number of molecules of OCIF or an analogue or variant thereof present in said complex as measured by an immunological assay technique (e.g. ELISA) to the number of molecules of OCIF or an analogue or variant thereof present in said complex as measured by a technique for measuring the total amount of protein present in said complex [e.g. Lowry's method: Lowry, O.H. *et al*, J. Biol. Chem, **193**, 265-275 (1951), absorbance (λ 280 nm) silver staining or the BCA method].

The number of molecules of OCIF or an analogue or variant thereof present in said complex as measured by an immunological assay technique can be determined using, for example, ELISA. The antibodies for use in binding to the immobilized phase and for labeling with a reporter enzyme such as a peroxidase in ELISA are any antibodies to the OCIF or analogue or variant thereof of interest that are suitable for the purpose. For example, suitable antibodies for binding to the solid phase include OI-26 purified from a culture of a hybridoma producing antibody OI-26 (FERM BP-6421) and OI-19 purified from a culture of a hybridoma producing antibody OI-19 (FERM BP-6420), while suitable antibodies for use as the antibody labeled with a

reporter enzyme in the mobile phase include anti-human OCIF monoclonal antibody OI-4 purified from a culture of a hybridoma producing antibody OI-4 (FERM BP-6419) labeled with peroxidase. A typical procedure for measuring the number of molecules of OCIF or an analogue or variant thereof in a complex is as follows:

- (a) Known concentrations of the free, uncomplexed OCIF are used to produce a calibration curve.
- (b) An ELISA is performed on the complex of interest and the calibration curve is then used to determine the concentration of OCIF.
- (c) Using the information obtained in (b) and the molecular weight of the OCIF monomer the number of molecules of OCIF in the tested complex is calculated.

The number of molecules of OCIF or an analogue or variant thereof present in said complex as measured by a technique for measuring the total amount of protein present in said complex can be determined using, for example Lowry's method. A typical procedure is as follows:

- (a) Known concentrations of bovine serum albumin are used to produce a calibration curve.
- (b) Lowry's method is then used to determine the total concentration of protein in the complex to be tested, the calibration curve being used to determine the concentration of OCIF.
- (c) Using the information obtained in (b) and the molecular weight of the OCIF monomer, the number of molecules of OCIF in the tested complex is calculated.

The actual ratio varies depending upon the type of immunoassay technique used and/or the technique used to measure the total protein. A preferred embodiment of the present invention utilises a complex of a human-originating OCIF or an analogue or variant thereof with dextran sulfate, wherein the ratio of the number of molecules of said OCIF or analogue or variant thereof present in said complex as determined by enzyme-linked immunosorbent assay (ELISA) using anti-human OCIF monoclonal antibody OI-19 purified from a culture of a hybridoma producing antibody OI-19 (FERM BP-6420) as the antibody bound to the solid phase and anti-human OCIF monoclonal antibody OI-4 purified from a culture of a hybridoma producing antibody OI-4 (FERM BP-6419) labeled with peroxidase in the mobile

phase to the number of molecules of OCIF or analogue or variant thereof present in said complex as determined by measuring the total protein content using Lowry's method is at least 0.5 but not greater than 1.2. More preferably, the ratio is at least 0.6 but not more than 1.1, and most preferably the ratio is at least 0.7 but not more than 1.1.

Preferred complexes for use in the present invention include the following:

- (a) a complex wherein said substance selected from the group consisting of OCIF, analogues thereof and variants thereof is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions and said polysaccharides and derivatives thereof are selected from the group consisting of hyaluronic acid, chondroitin sulfuric acid, dermatan acid, heparan acid, keratan acid, carrageenan, pectin, heparin, dextran and derivatives thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said substance selected from the group consisting of polysaccharides and derivatives thereof being from 1:1 to 1:10;
- (b) a complex wherein said substance selected from the group consisting of OCIF, analogues thereof and variants thereof is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions and said polysaccharides and derivatives thereof are selected from the group consisting of dextran sulfate and salts thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said substance selected from the group consisting of polysaccharides and derivatives thereof being from 1:1 to 1:10;
- (c) a complex wherein said substance selected from the group consisting of OCIF, analogues thereof and variants thereof is human monomeric or dimeric OCIF in which said monomer or one of the units of said OCIF dimer comprises amino acids +1 to +380 of SEQ. ID. NO.1 of the sequence listing and said polysaccharide derivative is a sodium salt of dextran sulfate having an average molecular weight of

from 1500 to 12000, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate being from 1:1 to 1:10;

(d) a complex according to (c) wherein the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate being from 1:1 to 1:8;

(e) a complex according to (c) wherein the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate being from 1:1 to 1:6; and

(f) a complex according to any one of (c) to (e) wherein said polysaccharide derivative is a sodium salt of dextran sulfate having an average molecular weight of from 1800 to 6000.

The complexes employed in the present invention can be prepared using any suitable method that favours binding of the polysaccharide or variant thereof to the OCIF or analogue or variant thereof. In its most general form, the method for the preparation of a complex for use in the present invention comprising at least one substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof, comprises the steps of incubating said at least one substance selected from the group consisting of OCIF, analogues thereof and variants thereof with said at least one substance selected from the group consisting of polysaccharides and derivatives thereof under conditions favouring the formation of a complex between said OCIF, analogues thereof or variants thereof and said polysaccharides or variants thereof and then removing any free polysaccharides or derivatives thereof that are not bound to said OCIF, analogues thereof or variants thereof.

The incubation of said at least one substance selected from the group consisting of OCIF, analogues thereof and variants thereof with said at least one substance selected from the group consisting of polysaccharides and derivatives

thereof is performed under any suitable conditions, but typically the incubation takes place under aqueous conditions. Preferably, the incubation is performed under alkaline conditions. More preferably, the incubation is performed at a pH of from 9.5 to 12. Most preferably, the incubation is performed at a pH of from 10 to 11.

During incubation, the range of the concentration of said OCIF, analogue or variant thereof in the aqueous solution is not particularly limited, as long as it is suitable to enable formation of the desired complex. Typically, the maximum concentration of said OCIF, analogue or variant thereof in the aqueous solution is from 0.1 to 0.5 mM and the minimum concentration is from 0.001 to 0.05 mM. Preferably, the concentration of said OCIF, analogue or variant thereof in the aqueous solution is from 0.01 to 0.2 mM, and most preferably it is from 0.05 to 0.1 mM. In the case of OCIF per se, the maximum concentration in the aqueous solution is from 10 to 50 mg/ml and the minimum concentration is from 0.1 to 5 mg/ml. Preferably, the concentration of OCIF in the aqueous solution is from 1 to 20 mg/ml, and more preferably it is from 5 to 10 mg/ml.

During incubation, the range of the concentration of said polysaccharide or variant thereof in the aqueous solution is not particularly limited, as long as it is suitable to enable formation of the desired complex. Typically, the maximum concentration of said polysaccharide or derivative thereof in the aqueous solution is from 0.1 to 0.5 M and the minimum concentration is from 0.00005 to 0.05 M. Preferably, the concentration of said polysaccharide or derivative thereof in the aqueous solution is from 0.005 to 0.25 M, and more preferably it is from 0.05 to 0.1 M. In the case of dextran sulfate sodium salt sulfur 5, the maximum concentration of said polysaccharide or variant thereof in the aqueous solution is from 200 mg/ml to 1000 mg/ml, and the minimum concentration is from 0.1 to 100 mg/ml. Preferably, the concentration of said polysaccharide or variant thereof in the aqueous solution is from 10 to 500 mg/ml and most preferably it is from 100 to 200 mg/ml.

During incubation, the temperature is not particularly limited, as long as it is suitable to enable formation of the desired complex. Typically, the upper limit of temperature for the incubation is from 37 to 50 °C, and the lower limit thereof is from 0 to 4 °C. Preferably, the temperature range is from 4 to 37 °C, and most preferably

the temperature range is from 4 to 10 °C.

As noted above, the complex used in the present invention does not comprise free polysaccharides or variants thereof which are not bound to OCIF, or an analogue or variant thereof. The method used to remove the free polysaccharides and variants thereof is not limited, as long as it is a method that is conventionally employed in procedures such as purification, isolation and/or fractionation. Examples of suitable methods include ion exchange chromatography, adsorption chromatography, partition chromatography, gel filtration chromatography, hydrophobic chromatography, affinity chromatography, crystallization, salting out and ultrafiltration. Of these, gel filtration chromatography (hereinafter referred to as "gel filtration") and ultrafiltration are preferred and gel filtration is most preferred.

There is no particular limitation on the gel used for the gel filtration for removal of free polysaccharides or variants thereof from the desired complex after incubation as long as it can be used for separation of the fraction containing the desired complex from the free polysaccharide or variants thereof which are not bound to the OCIF. Suitable examples include agarose gel, dextran gel and polyacrylamide gel.

The complexes used in the present invention comprising at least one substance selected from the group consisting of OCIF, analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof, can be distinguished from the free, uncomplexed OCIF or analogue or variant thereof per se using various measures including isoelectric point, sugar content and immunological detection.

The isoelectric point can be measured using any conventional isoelectric electrophoresis technique well-known to the skilled person in the art. OCIF is a basic protein and the isoelectric point thereof is about pI 9. This is significantly higher than that of the complexes that are employed in the present invention comprising OCIF and polysaccharides and variants thereof such as dextran sulfate, typical pI values of which are in the region of 5 to 7. Therefore, it is possible to readily distinguish complexed and uncomplexed OCIF using this technique.

The sugar content of the complexes used in the present invention and of free, uncomplexed OCIF or an analogue or variant thereof can be measured using any technique conventionally used to quantify neutral sugar content, typical examples including the phenol sulfuric acid method [M. Dubois et al., *Anal. Chem.*, **28**, 350 (1956)]. Since the total sugar content of a complex employed in the present invention comprising OCIF or an analogue or variant thereof and a polysaccharide or a variant thereof is greater than that of OCIF itself, they can be distinguished from each other.

A further alternative method for distinguishing free, uncomplexed OCIF or an analogue or variant thereof from the complexes that are used in the present invention comprising said OCIF or an analogue or variant thereof bound to a polysaccharide or a variant thereof is to quantify the amount of polysaccharide or variant thereof in each using an antibody which specifically binds to said polysaccharide or variant.

In order to measure the amount of protein in an OCIF or an analogue or variant thereof or in a complex used in the present invention comprising OCIF or an analogue or variant thereof and a polysaccharide or variant thereof, any technique conventionally used to measure total protein content can be used. Suitable examples include Lowry's method [Lowry, O.H. *et al*, *J. Biol. Chem*, **193**, 265-275 (1951)], absorbance (λ 280 nm) silver staining and the BCA method.

Free, uncomplexed OCIF or an analogue or variant thereof, or OCIF or an analogue or variant thereof present in the complex employed in the present invention can be measured immunologically using a method that employs at least one anti-OCIF monoclonal antibody. Examples of a suitable anti-OCIF monoclonal antibody preferably used for the immunological measurement of human OCIF include an antibody produced by hybridoma OI-19 (FERM BP-6420), an antibody produced by hybridoma OI-4 (FERM BP-6419) and an antibody produced by hybridoma OI-26 (FERM BP-6421) (e.g. see WO-A-99/15691). These antibodies are referred to as "antibody OI-19", "antibody OI-4", and "antibody OI-26", respectively, in the present invention. The antibody OI-19 and antibody OI-4 bind both OCIF monomer and OCIF dimer at an equivalent affinity, while antibody OI-26 specifically binds the

OCIF dimer. Immunological measurement can be performed using antibodies of this type according to any method well-known to the person skilled in the art (e.g. see WO-A-99/15691). Examples of suitable methods include enzyme immunoassay (referred to as "EIA"), radio immunoassay, enzyme-linked immunosorbent assay (ELISA) and sandwich EIA. Of these, ELISA is preferred. Where the OCIF is of human origin, ELISA can preferably be employed using antibody OI-19 or antibody OI-26 as the immobilized antibody and antibody OI-4 as the enzyme-labeled antibody. The preferred enzyme used for labeling the antibody is peroxidase (referred to as "POD").

Hybridoma producing antibody OI-4 was deposited domestically as "OI-4" at the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology at 1-3, Higashi 1 chome, Tsukuba-shi Ibaraki-ken 305-8566 Japan (which has since become the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology at AIST Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken 305-8566 Japan) on 16 October 1997 (Heisei-9) and a deposition number FERM P-16473 was granted. It was transferred to an international deposition with the deposition number FERM BP-6419 on 13 July 1998 (Heisei-10).

Hybridoma producing antibody OI-19 was deposited domestically as "OI-19" at the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology at 1-3, Higashi 1 chome, Tsukuba-shi Ibaraki-ken 305-8566 Japan (which has since become the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology at AIST Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken 305-8566 Japan) on 16 October 1997 (Heisei-9) and a deposition number FERM BP-16474 was granted. It was transferred to an international deposition with a deposition number FERM BP-6420 on 13 July 1998 (Heisei-10).

Hybridoma producing antibody OI-26 was deposited domestically as "OI-26" to National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology at 1-3, Higashi 1 chome, Tsukuba-shi Ibaraki-ken 305-8566 Japan (which has since become the International Patent Organism Depositary,

National Institute of Advanced Industrial Science and Technology at AIST Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken 305-8566 Japan) on 16 October 1997 (Heisei-9) and a deposition number FERM P-16475 was granted. It was transferred to an international deposition with a deposition number FERM BP-6421 on 13 July 1998 (Heisei-10) (see WO-A-99/15691).

The blood or serum concentration of complexes used in the present invention comprising OCIF or an analogue or variant thereof and a polysaccharide or a variant thereof can typically be measured as follows. First, said complex is administered together with at least one substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins in accordance with the present invention to a human or non-human animal. Then, after a defined length of time, blood or serum is recovered therefrom. The blood or serum concentration of said complex is then measured by ELISA using at least one anti-OCIF monoclonal antibody as described elsewhere in the present application (see WO-A-99/15691).

In the present invention, the complex of OCIF or an analogue or variant thereof and a polysaccharide or a variant thereof is used in combination with at least one substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins. Examples of said substances that suppress the production of prostaglandins and/or that compete with a biological action of prostaglandins that are suitable for use in the present invention include non-steroidal anti-inflammatory drugs (referred to hereinafter as NSAIDs), steroids, prostaglandin receptor antagonists, prostaglandin repressors, antibodies that neutralize a biological activity of prostaglandins, antibodies that neutralize the activity of prostaglandin receptors, substances that suppress the production of inflammatory cytokines that promote or induce the production of prostaglandins, substances that suppress the action of or compete with the action of inflammatory cytokines that promote or induce the production of prostaglandins, antibodies that neutralize the activity of inflammatory cytokines that promote or induce the production of prostaglandins, and antibodies that neutralize the activity of acceptors for inflammatory cytokines that promote or induce the production of prostaglandins. Of these, NSAIDs, steroids, and prostaglandin receptor antagonists are preferred, and NSAIDs are more preferred. NSAIDs act by inhibiting the action of prostaglandin

cyclooxygenase.

Preferred examples of NSAIDs suitable for use in the present invention include acidic non-steroidal drugs and basic non-steroidal drugs such as cyclooxygenase inhibitors (including selective cyclooxygenase-2 inhibitors), and the like.

Preferred examples of the acidic non-steroidal drugs include: loxoprofen sodium (Japanese Patent Publication No. 58-4699, DE-A-2814556 and US 4,161,538), indomethacin (US 3,161,654), mefenamic acid (US 3,138,636), ibuprofen (US 3,385,886), diclofenac sodium (US 3,558,690), naproxen (US 4,009,197), feprazone (US 3,703,528), pranoprofen (US 3,931,205), fentiazac (US 3,476,766), sulindac (US 3,654,349), clidanac (US 3,565,943), aspirin DL-lysine (FR-A-2115060), diflunisal (US 3,714,226), fenoprofen calcium (US 3,600,437), tiaprofenic acid (DE-A-2055264 and GB-A-1,331,505), tolfenamic acid (US 3,313,848), acetaminophen (US 3,910,952), piroxicam (US 3,591,584), emorfazone (JP 07-224030 and US 5,583,222), floctafenine (US 3,644,368), tenoxicam (DE-A-2537070 and GB-A-1,519,811), amfenac sodium (US 4,045,576), oxaprofen (US 3,578,671) and alminoprofen (US 3,957,850) (each of these is a non-selective COX inhibitor), and celecoxib [WO-A-95/15316, US 5,521,207 and Thomas D, P., et al., J. Med. Chem., 40, 1347-1365 (1997)], valdecoxib (US 5,859,257), meloxicam (US 4,233,299), tiracoxib (US 5,994,381), etoricoxib [British Medical Journal, 324, (7352) : 1488-92, Jun 2002], and rofecoxib (WO-A-95/00501 and US 5,474,995) (each of which is a selective inhibitor for COX-2). Preferred examples of the basic non-steroidal drugs include: mepirizole (ZA-A-6704936 and AU-A-6726083) and tiaramide hydrochloride (US 3,661,921) (each of these is a non-selective COX inhibitor).

Examples of suitable steroids for use in the present invention include: prednisolone (US 2,837,464), dexamethasone (US 3,007,923), betamethasone (US 3,053,865), and halopredone acetate (US 4,226,862); further examples can be found in Shimizu et al., Shikkantochiryō, the 3rd edition of revision, published by Nankoudō (1992). Examples of suitable prostaglandin receptor antagonists for use in the present invention include ONO-8711 [Watanabe, K., et al., Cancer Res., 59, p5093 (1999)] and ONO-8713 [Watanabe, K., et al, Cancer Lett, 156, p57 (2000)].

The most preferred substances that suppress the production of prostaglandins for use in the present invention are loxoprofen sodium and celecoxib.

The pharmaceutical compositions, agents and kits of the present invention comprising a combination of a complex of at least one substance selected from the group consisting of OCIF, analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof, and at least one substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins are useful for treating or preventing bone metabolic diseases. In the present invention, bone metabolic diseases are any diseases which are characterized by a decreased net amount of bone in the patient suffering therefrom and in which it is necessary to suppress bone resorption and/or the rate of bone resorption in order to treat or prevent said diseases.

Bone metabolic diseases that can be treated or prevented using the pharmaceutical compositions, agents and kits of the present invention include: bone metabolic diseases that can be treated or prevented by the complex of the present invention include: primary osteoporosis (senile osteoporosis, postmenopausal osteoporosis and idiopathic juvenile osteoporosis); endocrine osteoporosis (hyperthyroidism, hyperparathyroidism, Cushing's syndrome and acromegaly); osteoporosis accompanying hypogonadism (hypopituitarism, Klinefelter syndrome and Turner syndrome); hereditary and congenital osteoporosis (osteogenesis imperfecta, homocystinuria, Menkes syndrome, and Riley-Day syndrome); osteopenia due to gravity load mitigation or fixation and immobilization of limbs; Paget's disease; osteomyelitis; infectious focus due to loss of bone; hypercalcemia resulting from solid carcinoma (e.g. breast carcinoma, lung cancer, kidney cancer and prostatic cancer); a hemology-malignant disease (multiple myeloma, lymphoma and leukemia); idiopathic hypercalcemia; hypercalcemia accompanying hyperthyroidism or kidney malfunction; osteopenia resulting from steroid medication; osteopenia resulting from administration of other medicines (e.g. immunosuppressants such as methotrexate and cyclosporin A, heparin and antiepileptics); osteopenia resulting from kidney malfunction; osteopenia resulting from a surgical operation or digestive organ disease (e.g. small intestine hindrance, large intestine hindrance, chronic hepatitis,

gastrectomy, primary biliary liver cirrhosis and liver cirrhosis); osteopenia due to different types of rheumatism such as rheumatoid arthritis, osteoclasts; joint destruction due to different types of rheumatism such as rheumatoid arthritis; mucilage type rheumatism; osteoarthritis; loss of periodontal bone; cancer metastasis of bone (osteolysis metastasis); osteonecrosis or osteocyte death accompanying traumatic injury, Gaucher's disease, sickle cell anemia, systemic lupus erythematosus or nontraumatic injury; osteodystrophy such as renal osteodystrophy; osteopenia accompanying hypoalkalinephosphatasemia or diabetes; osteopenia accompanying nutritional disorders or eating disorders; other osteopenia; and cachexia due to solid carcinoma or cancer metastasis of bone or hemology-malignant disease (see, for example, Japanese Patent Application Publication 2000-178200).

The abovementioned pharmaceutical composition provided by the present invention may be orally or parenterally administered safely to human or non-human animals. The dosage form can be suitably selected and will vary depending on various factors such as the type of disease being treated, the extent of said disease, and the age, sex and weight of the patient. For example, it may be administered orally in the form of tablets, capsules, powders, granules or syrups, injected intravenously alone or in combination with conventional adjuncts such as glucose, amino acids or the like, injected intramuscularly, subcutaneously, intracutaneously or intraperitoneally alone, administered transdermally in the form of cataplasma, administered transnasally in the form of a nasal drop, administered transmucosally or to the oral cavity in the form of a mucous membrane applying agent, or administered intrarectally in the form of suppository. These preparations can be formulated in a conventional manner using well-known additives generally used in the field of medicine, such as excipients, binding agents, disintegrants, lubricants, flavoring agents, solubilizers, suspending agents, colorants, pH regulators, antiseptics, gelling agents, surfactants and coating agents.

When the pharmaceutical compositions of the present invention are in the form of a tablet, any carriers known in the art can be used. The carriers include, for example, excipients such as lactose, white sugar, sodium chloride, glucose, urea, starch, calcium carbonate, kaolin, crystalline cellulose, silicate or the like; binding agents such as water, ethanol, propanol, simple syrup, glucose solution, starch

solution, gelatin solution, carboxymethyl cellulose, shellac, methyl cellulose, potassium phosphate, polyvinyl pyrrolidone or the like; disintegrants such as dry starch, sodium alginate, agar powder, laminaran powder, sodium hydrogen carbonate, calcium carbonate, polyoxyethylene sorbitan fatty acid esters, sodium lauryl sulfate, stearic acid monoglyceride, starch, lactose or the like; decomposition inhibitors such as white sugar, stearin, cacao butter, hydrogenated oil or the like; absorption accelerators such as quaternary ammonium bases, sodium lauryl sulfate or the like; moisturizers such as glycerin, starch or the like; adsorbents such as starch, lactose, kaolin, bentonite, colloidal silicate or the like; and lubricants such as refined talc, stearic acid, metal salts of stearic acid such as calcium stearate and magnesium stearate, talc, boric acid powder, polyethylene glycol or the like. In addition, the tablet may be a tablet applied with general coatings, for example, sugar coating tablet, gelatin coating tablet, enteric coating tablet, film coating tablet, two layered tablet, multi-layered tablet or the like, if needed.

Where the compositions of the present invention are in the form of pills, the preparation may contain carriers known in the art, for example, excipients such as glucose, lactose, cacao butter, starch powder, hardened vegetable oil, kaolin, talc or the like; binding agents such as gum arabic powder, tragacanth powder, gelatin, ethanol or the like; and disintegrants such as laminaran, agar or the like.

Where the compositions of the present invention are in the form of a suppository, the preparation may contain conventional carriers such as polyethylene glycol, cacao butter, higher alcohols, esters of higher alcohols, gelatin, semi-synthesized glyceride or the like.

Where the compositions of the present invention are in the form of an injection, it is preferable that the preparation in the form of a solution or suspension is sterilized and is made isotonic with blood. When the preparations are in the form of a solution, an emulsion or a suspension, a variety of diluents known in the art can be used, including, for example, water, ethanol, propylene glycol, ethoxylated isostearyl alcohol, polyoxylated isostearyl alcohol, polyoxyethylene sorbitan fatty acid esters or the like. In addition, in such injectable formulations, the preparation may also contain salts, glucose, glycerin or the like in an amount sufficient to maintain

isotonicity with blood. They may also contain a solubilizer, a buffering agent, a soothing agent, a pH regulator, a stabilizer or a solubilizing agent. The injections can be freeze-dried after formulation.

Moreover, if needed, the preparations may also contain further additives such as a coloring agent, a preservative, a perfume, a flavoring agent, a sweetener or other medicines.

There is no specific limitation on the amount of the complex comprising at least one substance selected from the group consisting of OCIF, analogues thereof and variants thereof and at least one substance selected from the group consisting of polysaccharides and variants thereof that is present in the pharmaceutical composition of the present invention for administration in order to prevent or treat bone metabolic disease, but it is usually 0.1 to 70 % by weight, and preferably it is 1 to 30 % by weight of the whole formulation.

The dose of the complex comprising at least one substance selected from the group consisting of OCIF, analogues thereof and variants thereof and at least one substance selected from the group consisting of polysaccharides and variants thereof when used in combination with at least one substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins will vary depending on a variety of factors including the identity of said substance(s) that suppress the production of prostaglandins and/or that compete with a biological action of prostaglandins and the dose thereof, the condition to be treated, the age, sex and body weight of the patient and the administration route. However, the amount administered per day to an adult human is generally in a range having an upper limit of 1 to 50 mg/kg and a lower limit of 0.01 to 0.1 mg/kg. The more preferred range is 0.01 to 1 mg/kg per day, and the most preferred range is 0.1 to 1 mg/kg per day.

As noted above, when NSAIDs are used alone to try to treat or prevent bone metabolic diseases, they only have any effect when they are used in high doses that are higher than the effective dosage range for NSAIDs for the treatment or prevention of inflammation. One of the surprising discoveries in the present invention is that the complexes of OCIF, analogues thereof and variants thereof and polysaccharides

and variants thereof and the substances that suppress the production of prostaglandins and/or that compete with a biological action of prostaglandins show a remarkable synergistic effect in treating or preventing bone metabolic diseases. As a result, the effective dosage of said substances that suppress the production of prostaglandins and/or that compete with a biological action of prostaglandin in the pharmaceutical compositions is lower than that when used alone to treat or prevent bone metabolic diseases. In the pharmaceutical compositions of the present invention, the preferred dosage of said substances that suppress the production of prostaglandins and/or that compete with a biological action of prostaglandin is: (a) a dosage sufficient when administered alone to treat or prevent an inflammatory disease in the patient to be treated; and/or (b) a dosage that is not sufficient when administered alone to treat or prevent a bone metabolic disease in said patient.

The dose of the substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins when used in combination with the complex comprising at least one substance selected from the group consisting of OCIF, analogues thereof and variants thereof and at least one substance selected from the group consisting of polysaccharides and variants thereof depends on the identity of said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins, the identity of said complex and the dose thereof, the condition to be treated, the age, sex and body weight of the patient and the administration route. However, the amount administered per day to an adult human is generally in a range having an upper limit of 1 to 10 mg/kg and a lower limit of 0.01 to 0.1 mg/kg. The more preferred range is from 0.01 to 1 mg/kg per day, and the most preferred range is from 0.1 to 1 mg/kg per day.

In the case of the combined use of a complex of a substance selected from the group consisting of OCIF, analogues thereof and variants thereof and at least one substance selected from the group consisting of polysaccharides and variants thereof and loxoprofen sodium as the optimal substance that suppresses the production of prostaglandins, the dose of loxoprofen sodium is generally for an adult human in a range wherein the upper limit is 1 to 10 mg/kg per day and the lower limit is 0.01 to 0.1 mg/kg per day, and the preferred range is 0.01 to 1 mg/kg per day.

In the case of the combined use of a complex of a substance selected from the group consisting of OCIF, analogues thereof and variants thereof and at least one substance selected from the group consisting of polysaccharides and variants thereof depends and celecoxib as the optimal substance that suppresses the production of prostaglandins, the dose of celecoxib is generally for an adult human in a range wherein the upper limit is 5 to 50 mg/kg per day and the lower limit is 0.1 to 1 mg/kg per day, and the preferred range is 1 to 5 mg/kg per day.

In the case of the combined use of a complex of a substance selected from the group consisting of OCIF, analogues thereof and variants thereof and at least one substance selected from the group consisting of polysaccharides and variants thereof with a substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins, said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins may be administered to the patient before, at the same time as or after administering said complex to the patient. Said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins can be administered to the patient employing a similar method to that used for the administration of said complex, but it is not limited thereto.

Pharmaceutical compositions and agents for the prevention or treatment of bone metabolic disease suitable for the combined use of a complex of a substance selected from the group consisting of OCIF, analogues thereof and variants thereof and at least one substance selected from the group consisting of polysaccharides and variants thereof with a substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins are included in the pharmaceutical compositions and agents of the present invention. This is irrespective of the time of administration of said complex, the time of administration of said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins, the method by which each is administered or the like. Consequently, pharmaceutical compositions and agents suitable for said combined use according to the present invention can be in any well-known form conventionally used in the art for administering multiple active agents to

the same individual. Examples of such compositions include compounding agents, combined use agents and the like.

The pharmaceutical composition provided by the present invention can be administered once every several days, once per day or several times per day depending on the kind of the active ingredient contained in the pharmaceutical composition, the form of administration, the dosage form and the like. The agent for treatment or prevention of bone metabolic diseases can be administered once every several days, once per day or several times per day depending on the kind of the active ingredient contained in the pharmaceutical composition, the form of administration, the dosage form and the like.

The following preparation examples, reference examples and test examples are intended to further illustrate the present invention and are not intended to limit the scope of this invention in any way.

Preparation Example 1

Preparation of Complexes Comprising OCIF and Dextran Sulfate (I)

1(a) Preparation of Recombinant Dimeric Human OCIF

Recombinant dimeric human OCIF having a molecular weight of about 120000 was obtained according to the procedure described in examples of EP-A-0816380 (WO-A-96/26217). Namely, pBKOCIF, a plasmid vector comprising a nucleotide sequence that encodes human OCIF containing a signal peptide, obtained from the *E. coli* transformant strain pBK/01F10 [deposited as FERM BP-5267 under the Budapest Treaty at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology at 1-3, Higashi 1 chome, Tsukuba-shi Ibaraki-ken 305-8566 Japan (which has since become the International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology)] produced according to Example 11 of EP-A-0816380, was digested with restriction enzymes SalI and EcoRV. The nucleotide that encodes human OCIF containing a signal peptide, which is equivalent to human OCIF cDNA, was recovered according to the procedure described in Example 14 of EP-A-0816380. After separation and purification of said nucleotide, it was inserted into the expression vector pcDL-SR α 296 (Molecular and Cellular Biology, vol 8, p466, 1988), and then *E. coli* strain DH5 α (Gibco BRL), was transformed thereby (see the procedure described in Example 14 of EP-A-0816380). The recombinant vector named pSRaOCIF thus obtained was extracted from said transformant culture and purified.

The procedure of Example 14 of EP-A-0816380 was then applied to obtain the desired recombinant human mature OCIF. Namely, CHO dhFr- cells (ATCC, CRL 9096) were transfected with the recombinant plasmid pSRaOCIF produced above and a plasmid expressing dihydrofolate reductase (DHFR) (plasmid pBAdDSV disclosed in WO-A-92/01053) and then a DHFR-expressing transfectant was selected. The transformants that expressed large amounts of OCIF were cloned. The clones whose conditioned medium contained OCIF at a high concentration were selected and the clone expressing the largest amount of OCIF, 5561, was obtained. A culture of

clone 5561 thus obtained was conditioned and filtrated, and then applied to a Heparin Sepharose-FF column (2.6 x 10 cm, Pharmacia Co.) and subjected to column chromatography using a linear sodium chloride gradient as the eluant. The fraction having OCIF activity eluted with approximately 0.6 to 1.2 M sodium chloride was then applied to an affinity column (blue-5PW, 0.5 x 5.0 cm, Tosoh Co) and subjected to affinity chromatography using a linear sodium chloride gradient as the eluant. The eluted fractions were subjected to SDS-polyacrylamide gel electrophoresis under non-reducing conditions and the fractions containing the desired purified recombinant human mature OCIF were designated to show the band(s) with apparent molecular weights of 60000 and 120000 as shown in Example 14 of EP-A-0816380. The amino acid sequence of the monomeric peptide is shown in SEQ. ID. NO.1 of the sequence listing, which is identical with the full sequence of SEQ. ID. NO.4 or the amino acids No.1 to No.380 of SEQ. ID. NO.5 of WO-A-96/26217 and EP-A-0816380.

The combined fractions containing the obtained human OCIF was then supplemented with 1/100 volume of 25% trifluoroacetic acid and the resulting mixture was applied to a reverse phase column (PROTEIN-RP, 2.0 mm x 250 mm, purchased from YMC Co.) that had been pre-equilibrated with 30% acetonitrile containing 0.1% trifluoroacetic acid. The column was then eluted with a linear gradient of from 30% to 55% acetonitrile at a flow rate of 0.2 ml/min for 50 min. Two peak fractions were collected separately and then lyophilized. The fraction which showed a band having an apparent molecular weight of 120000 on SDS-PAGE under non-reducing conditions was then employed in the following examples as the dimeric human OCIF (see Examples 17 and 18 of WO-A-96/26217 and EP-A-0816380).

1(b) Preparation of Complexes Comprising OCIF and dextran sulfate

Purified dimeric human OCIF, prepared as described in Preparation Example 1(a) above, was dissolved in 10 mM sodium phosphate buffer solution (pH 6.0) containing 0.15 M sodium chloride to give solutions with an OCIF concentration of 1.5, 2, 5, 6.5, 10, 12.5, 20 or 50 mg/ml. Dextran sulfate sodium salt sulfur 5 (manufactured by Meito Sangyo Co., Ltd., hereinafter referred to as "DSS") was dissolved in the aqueous solutions thus produced to a final concentration of 40, 100,

130, 150, 200, 400, 500, 510 or 1000 mg/ml, and then 1 N sodium hydroxide was added thereto to a final pH of 10, 10.5 or 11. The obtained aqueous solutions were incubated at 4, 7, 25 or 37 °C for 1, 3, 6, 18, 24, 48, 72, 96, 144, 168 or 288 hours.

At the end of this incubation time, 4 ml of each resulting solution were applied to a Superdex 200 prep grade gel filtration column (inside diameter of the column: 16 mm; length: 60 cm, exclusion-limiting molecular weight: 1,300,000; manufactured by Amersham Bioscience) previously equilibrated with 10 mM sodium phosphate buffer (pH 6) containing 0.3 M sodium chloride, and then eluted with the same buffer at a flow rate of 2 ml/min. Absorption at wavelength 280 nm was monitored using an ultraviolet spectrophotometer, and the eluate at a retention time of about 28 to 36 minutes was collected. Free DS5 which had not bound to the OCIF was eluted at a retention time of about 50 to 70 minutes. All steps of this gel filtration procedure were performed at room temperature. The obtained preparations which contained the desired complexes of dimeric human OCIF and DS5 were frozen and stored at -60 °C. The preparation conditions for each complex are summarized in Table 1 below.

Table 1

Prep. number	DS5 conc. (mg/ml)	OCIF conc. (mg/ml)	Temp. (°C)	pH	Incubation Time (hours)
Preparation 1	130	6.5	4	10.5	18
Preparation 2	510	6.5	4	10.5	18
Preparation 3	130	6.5	4	11	18
Preparation 4	130	6.5	4	10.5	72
Preparation 5	500	5	4	10.5	144
Preparation 6	130	6.5	4	10.5	48
Preparation 7	130	6.5	4	10.5	144
Preparation 8	130	6.5	4	10.5	288
Preparation 9	400	20	4	10.5	18
Preparation 10	200	10	4	10.5	18
Preparation 11	100	5	4	10.5	18

Table 1 (contd.)

Prep. number	DS5 conc. (mg/ml)	OCIF conc. (mg/ml)	Temp. (°C)	pH	Incubation Time (hours)
Preparation 12	40	2	4	10.5	18
Preparation 13	1000	12.5	4	10.5	18
Preparation 14	1000	50	4	10.5	18
Preparation 15	400	2	4	10.5	144
Preparation 16	1000	5	4	10.5	18
Preparation 17	1000	2	4	10.5	18
Preparation 18	150	5	37	10.5	1
Preparation 19	150	5	37	10.5	3
Preparation 20	150	5	37	10.5	6
Preparation 21	150	5	37	10.5	24
Preparation 22	150	5	7	10.5	168
Preparation 23	150	5	4	10	144
Preparation 24	150	5	25	10	24
Preparation 25	130	6.5	4	10.5	24
Preparation 26	150	5	37	10	24
Preparation 27	150	5	4	10.5	144
Preparation 28	150	5	4	11	24
Preparation 29	150	5	4	10.5	24
Preparation 30	150	1.5	4	10.5	72
Preparation 31	130	6.5	25	10.5	1
Preparation 32	130	6.5	25	10.5	3
Preparation 33	130	6.5	25	10.5	6
Preparation 34	130	6.5	25	10.5	24
Preparation 35	130	6.5	25	10.5	168
Preparation 36	130	6.5	25	10.5	288
Preparation 37	150	5	4	10.5	96
Preparation 38	150	5	4	10.5	288
Preparation 39	130	6.5	25	10.5	18
Preparation 40	130	6.5	37	10.5	18

Preparation Example 2Preparation of Complexes Comprising OCIF and Dextran Sulfate (II)

Purified dimeric human OCIF, prepared as described in Preparation Example 1(a) above, was dissolved in 10 mM sodium phosphate buffer solution (pH 6.0) containing 0.15 M sodium chloride to give a solution having an OCIF concentration of 5 mg/ml. Dextran sulfate sodium salt having a molecular weight of 5000 (manufactured by Wako Pure Chemical Industries, Ltd., hereinafter referred to as "DS 5000") was dissolved in the aqueous solution thus obtained to give a final concentration of DS 5000 of 150 mg/ml, and then 1 N sodium hydroxide was added thereto to a final pH of 10.5. The aqueous solution thus obtained was incubated at 4 °C for 24 hours.

At the end of this incubation time, 4 ml of the resulting solution were applied to a Superdex 200 prep grade gel filtration column chromatography as described in Preparation Example 1(b) above. Absorption at wavelength 280 nm was monitored using an ultraviolet spectrophotometer, and the eluate at a retention time of about 28 to 36 minutes was collected. Free DS 5000 which had not bound to the OCIF was eluted at a retention time of about 40 to 65 minutes. The gel filtration was performed at room temperature.

The obtained preparation which contained the desired complex of dimeric human OCIF and DS5000 was frozen and stored at -60 °C. The preparation conditions for the complex are summarized in Table 2 below.

Table 2

Prep. number	DS5000 Conc. (mg/ml)	OCIF Conc. (mg/ml)	Temp. (°C)	pH	Incubation time (hours)
Preparation 41	150	5	4	10.5	24

Preparation Example 3

Measurement of Isoelectric Point

The purified recombinant dimeric human OCIF, prepared as described in Preparation Example 1(a) above and the complex of OCIF and dextran sulfate prepared in Preparation Example 1(b) above which is designated Preparation Number 22 in Table 1 were applied separately to an isoelectric electrophoresis gel IEF PAGE mini (pH range of 3 to 10, manufactured by Iwaki Glass), using an IEF pH 3-7 buffer kit (Technical Frontier Co.) and a voltage was applied to the gel according to the following regime: 100 V for 1 hour, followed by 200 V for 1 hour and finally 500 V for 30 minutes. After completion of the electrophoresis, the resultant gel obtained in each case was stained with Coomassie Blue.

From the electrophoresis gels obtained above, it was determined that the isoelectric point of the dimeric human OCIF was about pI 9, and the isoelectric point of the complex of OCIF and dextran sulfate designated Preparation Number 22 was about pI 6.5 by comparing the band position of OCIF and that of the OCIF complex with pI markers.

Preparation Example 4

Measurement of the Molecular Ratio of OCIF and Dextran Sulfate in a Complex

Comprising OCIF and Dextran Sulfate

4(a) Preparation of a Stock Solution of an Anti-Human OCIF Monoclonal Antibody OI-4 Labeled with Peroxidase

In this step, anti-human OCIF monoclonal antibody was labeled with peroxidase using an EZ-Link Maleimide Activated Horseradish Peroxidase Kit (manufactured by Pierce) according to the protocol II described in the instruction booklet supplied with the kit. Details of this procedure are as follows.

Anti-human OCIF monoclonal antibody OI-4 was purified from a culture of a hybridoma producing antibody OI-4 (FERM BP-6419) according to the method described in Example 4 of EP-A-0974671 (WO-A-99/15691), and then diluted to a final protein concentration of 1mg/ml with 10mM phosphate buffer (pH 7.6).

N-succinimidyl S-acetylthioacetate (provided in said EZ-Link Maleimide Activated Horseradish Peroxidase Kit) was dissolved in dimethylformamide to give a solution having a concentration of 10 mg/ml just before use. A 4 µl aliquot thereof was added to 1 ml of the diluted OI-4-containing solution prepared above, and the resulting solution was then incubated at room temperature for 30 minutes. At the end of this incubation time, 20 µl of a solution prepared just before use by dissolving 5 mg of hydroxylamine hydrochloride in 100 µl of Maleimide Conjugation Buffer (provided in said EZ-Link Maleimide Activated Horseradish Peroxidase Kit) were added thereto, and the resulting solution was incubated at a room temperature for 2 hours. At the end of this incubation time, the reaction mixture was applied to a polyacrylamide desalting column (10 ml, contained in said EZ-Link Maleimide Activated Horseradish Peroxidase Kit) previously equilibrated with 30 ml of Maleimide Conjugation Buffer (also provided in said kit), and then Maleimide Conjugation Buffer was applied to said column. The eluate was collected in 0.5 ml fractions. The 7th to 10th fractions containing the antibody were combined. 100 µl of a solution prepared just before use by dissolving 5 mg of EZ-Link Maleimide Activated Horseradish Peroxidase (contained in said EZ-Link Maleimide Activated Horseradish Peroxidase Kit) in 500 µl of distilled water were then added to the combined eluate fractions and the resulting mixture was incubated at room temperature for one hour. After incubation, an equal volume of glycerol was added thereto, and the solution thus obtained was stored at -20 °C.

The solution obtained by the above process was used as the stock solution of the anti-human OCIF monoclonal antibody OI-4 labeled with peroxidase (hereinafter referred to as "POD-OI-4"), and is referred to hereinafter as "POD-OI-4 stock solution".

4(b) Quantification of OCIF

The amount of OCIF present in any of the complexes prepared in Preparation Examples 1 and 2 above and the combination prepared in Reference Example 1 below was measured by enzyme-linked immunosorbent assay (ELISA) using two anti-OCIF monoclonal antibodies, the details of the procedure being as follows.

Anti-human OCIF monoclonal antibody OI-26 was purified from a culture of a hybridoma producing antibody OI-26 (FERM BP-6421) according to the method described in Example 4 of EP-A-0974671 (WO-A-99/15691), and then dissolved in 0.1 M sodium hydrogen carbonate to give a solution having a final protein concentration of 5 μ g/ml. A 100 μ l aliquot thereof was transferred to each well of a 96-well microtitre plate (Maxisorp, manufactured by NUNC), and the plate was then sealed and incubated at 4 °C overnight. At the end of this incubation time, each well was washed three times with 250 μ l of phosphate buffered saline (PBS) (pH 7.4) containing 0.1 % Polysorbate 20. 20 μ l of a dilution buffer solution [comprising 0.2 M Tris-hydrochloric acid, 40 % Block Ace (purchased from Dainippon Pharmaceutical Co., Ltd.), and 0.1% Polysorbate 20; pH 7.4] were added to each well, and then the plate was incubated at room temperature for 20 minutes to block areas of the well unbound by OI-26.

The samples to be added to the OI-26 bound wells prepared above were preferably diluted with the dilution buffer solution used above to block the wells. In order to make a calibration curve, the dilution buffer solution containing human OCIF at known concentrations was used as standards. The dilution buffer solution was used as a control. 50 μ l of each sample were transferred to each well.

After addition of the samples to the wells, 50 μ l of a solution obtained by diluting the POD-OI-4 stock solution [prepared as described in Example 4(a) above] 1500-fold volume with a dilution buffer solution [0.1 M Tris-hydrochloric acid, 40 % Block Ace (purchased from Dainippon Pharmaceutical Co., Ltd.), 0.1 % polysorbate 20 (pH 7.4)] were added to each well and the plate was then incubated at room temperature for 2 hours. At the end of this incubation time, each well was washed four times with 250 μ l of phosphate buffer containing 0.1 % polysorbate 20 (hereinafter referred to as "PB", pH 7.4).

0.1 M citric acid and 0.2 M disodium hydrogenphosphate were mixed, and used as a substrate solution (pH 4.5). A 32.5 ml aliquot thereof was transferred to a test tube and 6.5 μ l of hydrogen peroxide were added thereto. 13 mg of an o-phenylenediamine dihydrochloride (OPD) tablet (manufactured by Wako Pure

Chemical Industries, Ltd.) were then dissolved in the resulting solution. A 100 μ l aliquot thereof was added to each well, the plate was covered with aluminum foil, and then it was incubated at room temperature for 15 minutes. At the end of this incubation time, 50 μ l of the reaction stopping solution comprising purified water and concentrated sulfuric acid in a ratio of 250:50 by volume were added to each well. After stirring the solutions in the wells gently with a shaker (Titer mixer MB-1: manufactured by Japan Trika), the absorbance of each well at a wavelength of 490 nm was measured by a microplate reader (SPECTRA FLUOR: manufactured by TECAN).

On the basis of the calibration curve produced as explained above from the absorbance of standard solutions of human OCIF at known concentrations, the amount of human OCIF in each sample was calculated.

4(c) Quantification of Dextran Sulfate

The amount of dextran sulfate in each complex prepared in Preparation Examples 1 and 2 above was measured as the amount of neutral sugars by the phenol sulfuric acid method, the details of which are as follows.

A solution having a known concentration in the range of 10 to 60 μ g/ml of DS5 (manufactured by Meito Sangyo Co., Ltd.) or DS5000 (manufactured by Wako Pure Chemical Industries, Ltd.) was prepared using a diluting solution (0.01 M citric acid, 0.3 M sodium chloride, 0.01% polysorbate 80 aqueous solution: pH 6.0), and used as a standard solution. 0.2 ml each of the standard, a sample, or the diluting solution were transferred to each test tube. 0.2 ml of 50 mg/ml aqueous phenol were added thereto, and stirred rapidly. After incubating the resulting mixture at 60 °C for 20 seconds in a water bath, 1.0 ml of concentrated sulfuric acid was added thereto. After rapid stirring, the tube was incubated for 10 minutes at room temperature, stirred rapidly again, and then incubated for 20 minutes at room temperature. At the end of this incubation time, the absorbance of the solution in the tube at a wavelength of 490 nm was measured using a spectrophotometer (UV-240: manufactured by Shimadzu Seisakusho, K. K.).

From this absorbance and a calibration curve, the neutral sugar content was

determined. Human OCIF contains sugar chains. Therefore, the amount of dextran sulfate bound to human OCIF in the preparation being analysed was calculated by deducting the value of the neutral sugar content of human OCIF itself from that measured for any preparation being analysed.

4(d) Calculation of the Molecular Ratio of OCIF and Dextran Sulfate in a Complex Comprising OCIF and Dextran Sulfate

The amount of dextran sulfate present in the preparation being analysed, determined as described in Preparation Example 4(c) above was divided by the amount of human OCIF present in the preparation being analysed, determined as described in Preparation Example 4(b) above to give the amount of dextran sulfate present per 1 mg of human OCIF in the preparation being analysed.

The figure thus obtained was then used to calculate the molecular ratio of OCIF as monomer and dextran sulfate in the preparation being analysed by calculating the number of dextran sulfate molecules per one molecule of OCIF monomer, based on the assumption that the molecular weight of human OCIF monomer is 60000, the molecular weight of DS5 is 1950, the molecular weight of DS5000 is 5000.

The results obtained are shown in the following Table 3.

Table 3

Complex	Amount of dextran sulfate in the complex ($\mu\text{g}/\text{mg}$ OCIF)	Molecular ratio of OCIF as monomer and dextran sulfate in complex
Prep. 1	48.7	1:1.5
Prep. 2	100.2	1:3.1
Prep. 3	39.7	1:1.2
Prep. 4	62.0	1:1.9
Prep. 5	136.4	1:4.3
Prep. 6	60.7	1:1.9

Table 3 (contd.)

Complex	Amount of dextran sulfate in the complex ($\mu\text{g}/\text{mg}$ OCIF)	Molecular ratio of OCIF as monomer and dextran sulfate in complex
Prep. 7	58.5	1:1.8
Prep. 8	60.3	1:1.9
Prep. 9	67.7	1:2.1
Prep. 10	94.3	1:2.9
Prep. 11	63.6	1:2.0
Prep. 12	60.8	1:1.9
Prep. 13	144.9	1:4.5
Prep. 14	116.4	1:3.6
Prep. 15	126.9	1:4.0
Prep. 16	145.0	1:4.5
Prep. 17	116.5	1:3.6
Prep. 18	46.0	1:1.4
Prep. 19	61.0	1:1.9
Prep. 20	68.3	1:2.1
Prep. 21	110.7	1:3.4
Prep. 22	100.3	1:3.1
Prep. 23	65.8	1:2.1
Prep. 24	58.2	1:1.8
Prep. 25	43.8	1:1.4
Prep. 26	80.1	1:2.5
Prep. 27	61.8	1:2.0
Prep. 28	57.1	1:1.8
Prep. 29	69.3	1:2.2
Prep. 30	77.1	1:2.4
Prep. 31	34.5	1:1.1
Prep. 32	53.0	1:1.7
Prep. 33	47.4	1:1.5
Prep. 34	62.2	1:2.0

Table 3 (contd.)

Complex	Amount of dextran sulfate in the complex ($\mu\text{g}/\text{mg}$ OCIF)	Molecular ratio of OCIF as monomer and dextran sulfate in complex
Prep. 35	96.2	1:3.0
Prep. 36	122.5	1:3.9
Prep. 37	67.8	1:2.1
Prep. 38	69.5	1:2.4
Prep. 39	78.0	1:2.5
Prep. 40	98.4	1:3.1
Prep. 41	161.2	1:1.9

Preparation Example 5

The Stability of Binding Between OCIF and Dextran Sulfate in
OCIF/Dextran Sulfate Complexes

The gel filtration of a complex comprising OCIF and dextran sulfate was repeated twice, and the amount of dextran sulfate present in the complex obtained after each of said gel filtrations was measured. The details are as follows.

5(a) Incubation of OCIF and Dextran Sulfate

The procedure described above in Preparation Example 1(b) was used. Recombinant dimeric human OCIF, prepared as described in Preparation Example 1(a) above, was dissolved in 10 mM sodium phosphate buffer (pH 6.0) containing 0.15 M sodium chloride to give a solution having an OCIF concentration of 5 mg/ml. DS5 was dissolved in the solution thus obtained to give a final DS5 concentration of 150 mg/ml, and then 1 N sodium hydroxide solution was added thereto to adjust the pH to 10.5. The resulting solution was then incubated at 4 °C for 7 days to give a solution containing a complex of human dimeric OCIF and DS5.

5(b) First Gel Filtration

The solution containing a complex of human dimeric OCIF and DS5 obtained at the end of the incubation in Preparation Example 5(a) above was subjected

to gel filtration according to the method described in Preparation Example 1(b) above. The fractions at a retention time of about 28 to 36 minutes were collected, while free dextran sulfate which was not bound to OCIF was eluted at a retention time of about 50 to 70 minutes.

5(c) Measurement of Protein Content

The amount of protein present in the complex was measured according to Lowry's method [Lowry, O.H. *et al*, J. Biol. Chem, **193**, 265-275 (1951)] as follows.

0.2 g of copper (II) sulfate pentahydrate (Wako Pure Chemical) were dissolved in water to a final volume of 50 ml. 0.4 g of sodium tartrate dihydrate (Wako Pure Chemical) were dissolved in water to a final volume of 50 ml. 20 g of sodium carbonate were dissolved in water to a final volume of 100 ml. The three aqueous solutions thus obtained were mixed in a ratio of 1:1:2 by volume just before use (the resulting solution was referred to as the "A solution"). 10 g of sodium dodecyl sulfate (Nacalai Tesque Inc.) were dissolved in water to a final volume of 200 ml (the resulting solution was referred to as the "B solution"). 3.2 g of sodium hydroxide (Wako Pure Chemical) were dissolved in water to a final volume of 100 ml (the resulting solution was referred to as the "C solution"). A solution, B solution and C solution were mixed at a ratio of 1:2:1 by volume just before use.

Folin-ciocalteu reagent (Wako Pure Chemical) and water were mixed in a ratio of 1:5 by volume just before use. 2.76 g of citric acid, trisodium salt dihydrate (Wako Pure Chemical), 0.13 g of citric acid monohydrate (Wako Pure Chemical), 17.5g of sodium chloride and 0.1 g of polysorbate 80 were dissolved in water to a final volume of 1 L (pH 6.9) to give a solution referred to as the "diluting solution".

9.5 ml of diluting solution were added to 500 μ L of a standard solution of bovine serum albumin (Pierce Co. Ltd.) containing 2 mg/ml of bovine serum albumin (referred to as "BSA") in 0.9% aqueous sodium chloride containing sodium azide at a concentration of less than 0.1% to give a solution referred to as "100 μ g/ml BSA solution". 3.5 ml, 3 ml, 2.5 ml or 2 ml of diluting solution were added to 1.5 ml, 2 ml, 2.5 ml or 3 ml of 100 μ g/ml BSA solution, respectively to give solutions

referred to as "30 μ g/ml BSA solution", "40 μ g/ml BSA solution", "50 μ g/ml BSA solution" and "60 μ g/ml BSA solution" respectively. 3 ml of diluting solution were added to 1.5 ml of 60 μ g/ml BSA to give a solution referred to as "20 μ g/ml BSA solution".

The sample whose protein content was to be determined was diluted with diluting solution to give a solution with a final protein concentration of about 40 μ g protein per 1 ml. 1 ml of 20 μ g/ml BSA solution, 30 μ g/ml BSA solution, 40 μ g/ml BSA solution, 50 μ g/ml BSA solution, 60 μ g/ml BSA solution, the diluted sample or diluting solution (n=3) were transferred to a test tube, and 1 ml of alkaline copper reagent was added thereto, and the resulting solution was mixed and incubated at room temperature for 10 minutes. 0.5 ml of the diluted folin-ciocalteu reagent were then added thereto and the resulting solution was mixed and incubated at room temperature for 30 minutes. At the end of this incubation time, the absorbance of each mixture at a wavelength of 750 nm was measured using a cell made of quartz whose width was 10 mm using an ultraviolet spectrophotometer (Lambda 20: Perkin Elmer Co Ltd.). Then, the amount of protein contained in the sample was calculated on the basis of a calibration curve produced using the absorbances of the standard BSA solutions (as a value reduced to an amount of BSA).

5(d) Quantification of Dextran Sulfate

The amount of dextran sulfate bound to human OCIF in the complex that was obtained after the first gel filtration in Preparation Example 5(b) above was measured using the procedure described in Preparation Example 4(c) above.

5(e) Second Gel Filtration

The combined fractions that were obtained in Preparation Example 5(b) above were transferred to two Centriprep filter units (YM-30, 30,000 MW cutoff, Millipore Amicon Co Ltd.), and they were centrifuged at 2000 rpm for 20 min using a centrifuge machine (himacCT60, Hitachi Seisakusho Co Ltd.). The solutions concentrated by the two Centriprep filter units were collected and combined. The resulting solution was subjected to gel filtration as described in Preparation Example 1(b) above, and the fractions at a retention time of about 28 to 36 minutes were

collected and combined. Then, the protein and sugar content in the complex present in the combined fractions was measured as described in Preparation Examples 5(c) and 5(d) above.

5(f) Third Gel Filtration

The combined fractions that were obtained in Preparation Example 5(e) above were transferred to two Centriprep filter units (YM-30, 30,000 MW cutoff, Millipore Amicon Co Ltd.), and they were centrifuged at 2000 rpm for 20 min using a centrifuge machine (himacCT60, Hitachi Seisakusho Co Ltd.). The solutions concentrated by the two Centriprep filter units were collected and combined. The obtained concentrate was subjected to gel filtration as described in Preparation Example 1(b) above, and the fractions at a retention time of about 28 to 36 minutes were collected and combined. Then, the protein and sugar content in the complex present in the combined fractions was measured as described in Preparation Examples 5(c) and 5(d) above.

5(g) Calculation of the Molecular Ratio of OCIF to Dextran Sulfate

The molecular ratio of OCIF as monomer to dextran sulfate present in the complex contained in the fractions obtained after the first gel filtration in Preparation Example 5(b) above, the second gel filtration in Preparation Example 5(e) above and the third gel filtration in Preparation Example 5(f) above were calculated according to Preparation Example 4(d) above. The results obtained are summarized in Table 4 below.

Table 4

Gel Filtration	Molecular ratio of OCIF as monomer to dextran sulfate in the complex
First	1:2.2
Second	1:2.3
Third	1:2.1

It will be immediately apparent from the above that the molecular ratio of OCIF to dextran sulfate in the complex used in the present invention is remarkably

constant throughout the repeated gel filtrations, indicating the high degree of stability of the binding between OCIF and dextran sulfate in the complexes of the present invention.

Preparation Example 6

The Degree of Adsorption of a Complex of OCIF and Dextran Sulfate to a Heparin Cross-Linked Column

6(a) Heparin Column Chromatography

All the column chromatography procedures in this example were performed at a flow rate of 4 ml per minute.

A heparin cross-linked column (HiTrap Heparin HP column, Lot.289212, Amersham Bioscience) was pre-equilibrated with 5 ml of 10 mM sodium phosphate buffer containing 0.7 M sodium chloride. A preparation from Table 1 of Preparation Example 1 was taken and diluted to a final protein concentration of 0.1 mg/ml with 10 mM sodium phosphate buffer containing 0.7 M sodium chloride. 1 ml of the diluted solution thus obtained was applied to said column and 1 ml of a first eluate was collected (fraction A). Next, 5 ml of 10 mM sodium phosphate buffer containing 0.7 M sodium chloride were applied to said column and 5 ml of a second eluate were collected (fraction B). Finally, 4 ml of 10 mM sodium phosphate buffer containing 2M sodium chloride were applied to said column and 4 ml of an eluate were collected (fraction C).

6(b) Measurement of the Amount of OCIF in the Eluate

100 μ L of 0.1 M sodium hydrogen carbonate (pH 9.6), in which was dissolved an anti-human OCIF monoclonal antibody OI-19 (FERM BP-6420) at a concentration of 10 μ g per ml, were transferred to each well of a 96-well microtitre plate (Maxisorp: NUNC Co Ltd.). The plate was sealed and then incubated at 4 °C overnight. At the end of this incubation time, the solution in each well was removed by decantation, 300 μ L of 50% Block Ace (purchased from Dainippon Pharmaceutical Co., Ltd.) were added to each well, and then the plate was incubated at room temperature for 2 hours. After removing the solution, each well was washed three times with 300 μ L of PBS (pH 7.4) containing 0.1% polysorbate 20 using a

SERA WASHER MW-96R (Bio Tec Co Ltd.).

After preparing the wells as described above, 20 μ L of each of the three eluates (fractions A, B and C) obtained in Preparation Example 6(a) above were diluted to a final volume of 120 μ L with 0.2 M Tris-HCl (pH 7.4) containing 40% Block Ace, 10 μ g/ml of mouse immunoglobulin G and 0.1% polysorbate 20, and then diluted with the same volume of pure water. At the same time, a known amount of human OCIF was dissolved in 120 μ L of 0.2 M Tris-HCl (pH 7.4) containing 40% Block Ace, 10 μ g/ml of mouse immunoglobulin G and 0.1% polysorbate 20, and then diluted with the same volume of pure water. The solution thus obtained was used as a standard.

100 μ L of each of the diluted eluates and of the standard were added to one well each of the pre-prepared microtitre plate described above and then the plate was incubated at room temperature for 2 hours with gentle mixing using a microplate mixer (NS-P: Iuchi Seiei-Do, Co Ltd.). At the end of this incubation time, the solution was removed from each well, and then each well was washed six times with 300 μ L of PBS (pH 7.4) containing 0.1% polysorbate 20 using a SERA WASHER MW-96R (Bio Tec Co Ltd.). 100 μ L of 0.1 M Tris-HCl (pH 7.4) containing 25% Block Ace, 10 μ g/ml of mouse immunoglobulin G and 0.1% polysorbate 20, to which had been added the POD-OI-4 stock solution prepared in Preparation Example 4(a) above to give a 0.01% solution (volume per volume), were then added to each well and the plate was incubated at room temperature for 2 hours with gentle mixing using the same microplate mixer. After removing the solution, each well was washed six times with 300 μ L of PBS (pH 7.4) containing 0.1% polysorbate 20 using a SERA WASHER MW-96R (Bio Tec Co Ltd.).

After the wells were washed, 100 μ L of 3,3', 5,5'-tetramethylbenzidine (TMB) soluble reagent (Scytek Co Ltd.) were added to each well and the plate was then incubated at room temperature for 10 to 15 minutes with gentle mixing using the same microplate mixer as above. At the end of this incubation time, 100 μ L of TMB stop buffer (Scytek Co Ltd.) were added to each well. After mixing the plate

gently with the microplate mixer for about 1 minute, the absorbance of each well at a wavelength of 450 nm was measured using a microplate reader (SPECTRA THERMO : TECAN Co Ltd.). The amount of OCIF contained in each of fractions A, B and C [designated (a), (b) and (c)] was then calculated on the basis of a calibration curve prepared by plotting the absorbance of each standard described above against concentration. The degree of adsorption of the tested complex of OCIF and dextran sulfate to the heparin cross-linked column was then calculated according to the following formula:

$$\frac{(c)}{(a) + (b) + (c)}$$

The results are summarized in Table 5 below for the OCIF complexes Preparations 6-8, 22, 24, 25 and 27 prepared in Preparation Example 1 above. The corresponding result for non-complexed OCIF is also given. As can be seen from the table, non-complexed OCIF bound more strongly to the heparin column than the complex of the present invention. It was also found that the complexes of the present invention can be further characterized by their degree of adsorption to a heparin cross-linked column.

Table 5

Preparation	The degree of adsorption of the OCIF/DS complex to a heparin cross-linked column
Prep. 6	0.451
Prep. 7	0.183
Prep. 8	0.153
Prep. 22	0.264
Prep. 24	0.072
Prep. 25	0.611
Prep. 27	0.141
OCIF	0.998

Preparation Example 7Immunological Detection of an OCIF/Dextran Sulfate Complex7(a) Measurement of the Amount of Protein

The amount of protein contained in OCIF complexes Preparations 6-8, 22, 24, 25 and 27 prepared in Preparation Example 1 above was determined according to the method described in Preparation Example 5(c) above.

7(b) Immunological Measurement of the Amount of OCIF

The amount of OCIF contained in OCIF complexes Preparations 6-8, 22, 24, 25 and 27 prepared in Preparation Example 1 above determined by immunological means was determined by the ELISA technique described in Preparation Example 6 above.

7(c) Calculation of the Immunological Detection Rate

The value obtained in Preparation Example 7(b) above was divided by the corresponding value obtained in Preparation Example 7(a) above, and the resulting value thus obtained was referred to as "the immunological detection rate".

The results are summarized in Table 6 below. The corresponding result for non-complexed OCIF is also given. It was also found that the complexes of the present invention can be further characterized by their immunological detection rate.

Table 6

Preparation	The immunological detection rate of OCIF/DS complex.
Prep. 6	1.07
Prep. 7	0.74
Prep. 8	0.88
Prep. 22	1.06
Prep. 24	1.02
Prep. 25	0.87
Prep. 27	1.04
OCIF	1.06

Reference Example 1Preparation of a Combination of OCIF and Dextran Sulfate

A combination of OCIF and dextran sulfate sodium salt (molecular weight 5000 or 10000) was prepared as follows using the procedure disclosed in Example 1 of EP-A-1127578 (WO-A-2000/24416).

Purified dimeric human OCIF having a molecular weight of about 120000, prepared as described in Preparation Example 1(a) above, was dissolved in 10 mM sodium phosphate buffer solution (pH 6.0) containing 0.15 M sodium chloride and 0.01 % of polysorbate 80 to give a solution having an OCIF concentration of 0.25 mg/ml. DS 5000 (manufactured by Wako Pure Chemical Industries, Ltd.), described in Preparation Example 2 above or dextran sulfate sodium salt having a molecular weight of 10000 (manufactured by Wako Pure Chemical Industries, Ltd., hereinafter referred to as "DS10000") was dissolved in the resulting aqueous solution to give a solution having a final concentration of the dextran sulfate sodium salt of 1 or 4 mg/ml, and then sodium hydroxide was added thereto to give a final pH of 7. The aqueous solutions thus obtained were incubated at 4 °C for 24 hours to give the desired preparations containing OCIF and DS5000 or DS10000, which were then used for comparison purposes in Test Example 1 below.

The preparation conditions for each combination are summarized in Table 7 below.

Table 7

Ref. Prep. Number	Dextran sulfate		OCIF Conc. (mg/ml)	Temp. (°C)	pH	Incubation time (hours)
	type	Conc. (mg/ml)				
Ref.Prep.1	DS5000	4	0.25	4	7	24
Ref.Prep.2	DS10000	1	0.25	4	7	24

Test Example 1

Measurement of the Serum Concentration of Complexes Comprising OCIF and Dextran Sulfate

1(a) Injection and Blood Collection

Five-week old Wistar female rat (having a body weight of about 100 g) were made to abstain from food overnight. The preparation of OCIF and dextran sulfate prepared in either Preparation Example 1, Preparation Example 2 or Reference Example 1 which was to be tested was diluted to a concentration of 0.25 mg/ml with PBS (pH 7.4) containing 0.01 % Polysorbate 80 to prepare an injectable solution, which was then administered to the tail of one of the test rats via a vein in a single dose at an injected level of 2 ml/kg body weight. 6 hours after administration, blood was taken from the heart of the rat.

1(b) Fractionation of Serum

After allowing the blood collected in 1(a) above to coagulate at room temperature for 30 minutes, serum was obtained therefrom as a supernatant by centrifugation of the blood at 14000 rpm for 3 minutes using a rotor with a diameter of 10 cm.

1(c) Quantification of OCIF in the Serum

100 µl of a solution wherein anti-human OCIF monoclonal antibody OI-19 (see EP-A-0974671/WO-A-99/15691) were dissolved in 0.1 M sodium hydrogen carbonate solution to a final OCIF concentration of 10 µg/ml were added to each well of a 96-well micro titre plate (Maxisorp: manufactured by NUNC), and then the plate was sealed and allowed to stand overnight at 4 °C. The antibody solution was then removed by decantation, and 300 µl of a blocking buffer solution (50 % Block Ace: purchased from Dainippon Pharmaceutical Co., Ltd.) were added to each well and then the plate was allowed to stand at room temperature for 2 hours. At the end of this incubation time, each well was washed three times with 300 µl of PBS (pH 7.4) containing 0.1 % Polysorbate 20.

100 µl of purified water and 120 µl of a dilution buffer solution
[composition: 0.2 M Tris-hydrochloric acid, 40 % Block Ace (purchased from

Dainippon Pharmaceutical Co., Ltd.), 10 µg/ml mouse immunoglobulin G, and 0.1 % polysorbate 20: pH 7.4] were added to 20 µl of the serum to be tested that was collected as described in 1(b) above, and mixed. As a control, 100 µl of purified water and 120 µl of dilution buffer containing human OCIF dimer at a known concentration were added to 20 µl of distilled water, and mixed.

100 µl of each of the serum preparations thus obtained were added to each well, and the plate was then allowed to stand at room temperature for 2 hours. Each well was washed six times after the reaction was complete with 300 µl of a solution containing 0.1% Polysorbate 20 (pH 7.4). 100 µl of a solution obtained by diluting 1000-fold the POD-OI-4 stock solution obtained in Preparation Example 4(a) above with a dilution solution [comprising 0.1 M Tris-hydrochloric acid, 25% Block Ace (purchased from Dainippon Pharmaceutical Co., Ltd.), 10 µg/ml mouse immunoglobulin G and 0.1 % Polysorbate 20 (pH 7.4)] were then added to each well, and the plate was allowed to stand at room temperature for 2 hours.

At the end of this incubation time, each well was washed six times with 300 µl of PBS (pH 7.4) containing 0.1 % Polysorbate 20. 100 µl of a substrate solution (TMB soluble reagent: manufactured by Scytek) were then added to each well, and the plate was allowed to stand at room temperature for 10 to 15 minutes. 100 µl of a reaction stop solution (TMB stop buffer: manufactured by Scytek) were then added to each well.

After stirring gently using a shaking machine (Micro plate mixer NS-P: manufactured by Iuchi Seiei-Do Co Ltd.), the absorbance of each well at a wavelength of 450 nm was measured using a micro plate reader (SPECTRA THERMO: manufactured by TECAN). The OCIF concentration in the tested serum was then calculated from a calibration curve created using the standard OCIF solution. The dose was calculated as the dose of OCIF per kg body weight (mg/kg) by measuring the concentration of OCIF in each injection prepared in 1(a) in a similar manner to the case of the serum.

1(d) Serum Concentration

The OCIF in the serum obtained in 1(b) above was quantified for each

sample according to the method described in 1(c) above. The results are shown in the Table 8 below.

Table 8

Preparation	Dose (OCIF mg/kg)	Serum concentration (OCIF ng/ml)	Corrected serum concentration* (OCIF ng/ml)
Prep. 1	0.5	213	
Prep. 2	0.5	350	
Prep. 3	0.5	191	
Prep. 4	0.5	370	
Prep. 5	0.5	305	
Prep. 6	0.5	209	
Prep. 7	0.5	371	
Prep. 8	0.5	571	
Prep. 9	0.5	164	
Prep.10	0.5	174	
Prep. 11	0.5	235	
Prep. 12	0.5	249	
Prep. 13	0.5	177	
Prep. 14	0.5	271	
Prep. 15	0.5	313	
Prep. 16	0.5	359	
Prep. 17	0.5	269	
Prep. 18	0.6	400	351
Prep. 19	0.4	526	614
Prep. 20	0.4	553	760
Prep. 21	0.1	132	611
Prep. 22	0.6	752	651
Prep. 23	0.5	340	
Prep. 24	0.5	830	
Prep. 25	0.5	165	
Prep. 26	0.5	574	
Prep. 27	0.5	584	

Table 8 (contd.)

Preparation	Dose (OCIF mg/kg)	Serum concentration (OCIF ng/ml)	Corrected serum concentration* (OCIF ng/ml)
Prep. 28	0.5	228	
Prep. 29	0.5	231	
Prep. 30	0.5	620	
Prep. 31	0.5	338	
Prep. 32	0.5	774	
Prep. 33	0.5	879	
Prep. 34	0.5	667	
Prep. 35	0.2	318	795
Prep. 36	0.1	114	570
Prep. 37	0.5	535	
Prep. 38	0.4	631	789
Prep. 39	0.5	366	
Prep. 40	0.5	423	
Prep. 41	0.4	423	508
Ref.Prep. 1	0.5	75	
Ref.Prep. 2	0.5	24	

* Corrected concentration in serum is the OCIF concentration in serum when converting the dose of OCIF per kg body weight to 0.5 mg/kg.

As shown in Table 8, the serum concentrations of the preparations of the present invention administered at a dose of 0.5 mg/kg body weight six hours after administration were 2.2 to 11.7 times higher than that obtained after administration of Reference Preparation 1 with the same dose.

Test Example 2

Combined Use of a complex of OCIF and dextran sulfate and a NSAID (1)

This example was carried out according to the method described in C. V. Winder, et al. Arthritis Rheum **12** 472 (1957).

Heat-killed cells of *Mycobacterium butyricum* were pulverized in an agate mortar and then suspended in liquid paraffin sterilized with hot air to give a suspension having a cellular concentration of 2 mg/ml. This suspension was then subjected to ultrasonication so as to produce an adjuvant.

50 μ l of the obtained adjuvant were injected intradermally at each of two sites in the base of the tail of a Lewis rat (eight-weeks old, female) (200 μ g in total per rat), to induce arthritis in said rat.

From the 14th to 20th days after injection of the adjuvant, loxoprofen sodium (Japanese Patent Publication No. 58-4699) suspended in a 0.5 % tragacanth aqueous solution (manufactured by Nihon Funmatsu Yakuhin Co., Ltd.) at a concentration of 1 mg/ml was orally administered to each rat of one test group at a rate of 5 ml/kg each day so that the dose of loxoprofen sodium administered was 5 mg/kg body weight. The OCIF complex Preparation 11 prepared as described in Preparation Example 1 above, that had been pre-dissolved in physiological saline (manufactured by Otsuka Pharmaceutical Co., Ltd.) containing 0.1 mg/ml of Tween 80 (manufactured by Sigma) to a concentration of the OCIF complex of 0.05 mg/ml was administered intraperitoneally each day from the 14th to 20th days after injection of the adjuvant at a rate of 2 ml/kg to each rat of a second test group so that the dose of OCIF complex was 0.1 mg/kg body weight. To each rat of a third test group, the same 0.5 % tragacanth aqueous solution of loxoprofen sodium as administered to the first test group was orally administered at a loxoprofen sodium dose of 5 mg/kg body weight and the same saline solution of OCIF complex Preparation 11 described in Preparation Example 1 above as administered to the second test group was administered at a dose of 0.1 mg/kg body weight each day from the 14th to 20th days after injection of the adjuvant in the same manner as above. As a control, instead of the loxoprofen sodium, OCIF complex Preparation 11 or a combination thereof, only the solvents used for dissolving them were administered to the rat.

Each rat was dissected on the 21st day after injection of the adjuvant, and the right-and-left femurs were extracted. The bone mineral densities of the distal ends

of the right-and-left femurs were measured using an X-ray measurement apparatus (Dual energy X-ray absorptiometry DCS-600R, manufactured by Aroka Co., Ltd.), and the average of each of the measured values was calculated, and was defined as the bone mineral density of the individual.

In order to evaluate the differences between the averages of the bone mineral densities of each group, significant difference was tested by Student's t-test, and the P value was calculated. The results are as is shown in Table 9.

Table 9

Administered Preparation	Bone mineral density (mg/cm ²) (average \pm standard error)
Solvent	106.4 \pm 1.0
Loxoprofen sodium	106.5 \pm 0.8
Preparation 11	109.6 \pm 2.1
Loxoprofen sodium and Preparation 11	115.2 \pm 2.1

- 1) The P value for the difference of the bone mineral density of the group to which the solvent was administered and that of the group to which loxoprofen sodium and Preparation 11 was administered was less than 0.01.
- 2) The P value for the difference of the bone mineral density of the group to which loxoprofen sodium was administered and that of the group to which loxoprofen sodium and Preparation 11 were administered was less than 0.01.

As shown in Table 9, the improvement in adjuvant-arthritis resulting from the combined use of loxoprofen sodium and OCIF complex Preparation 11 was significantly superior not only to that obtained where solvent alone was administered but also to the improvement obtained in the two test groups to which loxoprofen sodium and OCIF complex Preparation 11 were administered alone. Indeed, it will be readily appreciated that the combined effect of the administration of the loxoprofen sodium and OCIF complex Preparation 11 was super-additive, i.e. the combination exhibited synergy.

Test Example 3

Combined Use of a complex of OCIF and dextran sulfate and a NSAID (2)

Arthritis was induced in test groups of Lewis rats (eight-weeks old, female) according to the same procedure described in Example 2 above.

From the 14th to 20th days after injection of the adjuvant, celecoxib [Thomas D, P., et al., J. Med. Chem., **40**, 1347-1365 (1997); WO-A-95/15316] suspended in a 0.5 % tragacanth aqueous solution (manufactured by Nihon Funmatsu Yakuhin Co., Ltd.) at a concentration of 0.2 mg/ml was orally administered to each rat of one test group at a rate of 5 ml/kg each day so that the dose of loxoprofen sodium administered was 1 mg/kg body weight. The OCIF complex Preparation 22 prepared as described in Preparation Example 1 above, that had been pre-dissolved in physiological saline (manufactured by Otsuka Pharmaceutical Co., Ltd.) containing 0.1 mg/ml of Tween 80 (manufactured by Sigma) to a concentration of the OCIF complex of 1.75 mg/ml was administered intraperitoneally each day from the 14th to 20th days after injection of the adjuvant at a rate of 2 ml/kg to each rat of a second test group so that the dose of OCIF complex was 3.5 mg/kg body weight. To each rat of a third test group, the same 0.5 % tragacanth aqueous solution of celecoxib as administered to the first test group was orally administered at a celecoxib dose of 1 mg/kg body weight and the same saline solution of OCIF complex Preparation 22 described in Preparation Example 1 above as administered to the second test group was administered at a dose of 3.5 mg/kg body weight each day from the 14th to 20th days after injection of the adjuvant in the same manner as above. As a control, instead of the celecoxib, OCIF complex Preparation 22 or a combination thereof, only the solvents used for dissolving them were administered to the rat.

Each rat was dissected on the 21st day after injection of the adjuvant, and the right-and-left femurs were extracted. The bone mineral densities of the distal ends of the right-and-left femurs were measured using an X-ray measurement apparatus (Dual energy X-ray absorptiometry DCS-600R, manufactured by Aroka Co., Ltd.), and the average of each of the measured values was calculated, and was defined as the bone mineral density of the individual.

In order to evaluate the differences between the averages of the bone mineral

densities of each group, significant difference was tested by Student's t-test, and the P value was calculated.

The results are as is shown in Table 10.

Table 10

Administrated Preparation	Bone mineral density (mg/cm ²) (average \pm standard error)
Solvent	103.3 \pm 1.1
Celecoxib	103.0 \pm 1.8
Preparation 22	104.2 \pm 2.1
Celecoxib and Preparation 22	117.2 \pm 1.9

- 1) The P value for the difference of the bone mineral density of the group to which the solvent was administered and that of the group to which celecoxib and Preparation 22 were administered was less than 0.01.
- 2) The P value for the difference of the bone mineral density of the group to which celecoxib was solely administered and that of the group to which celecoxib and Preparation 22 were administered was less than 0.001.
- 3) The P value for the difference of the bone mineral density of the group to which Preparation 22 was solely administered and that of the group to which celecoxib and Preparation 22 were administered was less than 0.001.

As shown in Table 10, the improvement in adjuvant-arthritis resulting from the combined use of celecoxib and OCIF complex Preparation 22 was significantly superior not only to that obtained where solvent alone was administered but also to the improvement obtained in the two test groups to which celecoxib and OCIF complex Preparation 22 were administered alone. Indeed, it will be readily appreciated that the combined effect of the administration of the celecoxib and OCIF complex Preparation 22 was super-additive, i.e. the combination exhibited synergy.

Advantages Provided by the Invention

As demonstrated above, the OCIF in the pharmaceutical compositions and agents of the present inventions comprising (1) a complex of at least one OCIF, an analogue or a variant thereof and at least one polysaccharide or a derivative thereof, and (2) at least one substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins, is retained in the blood after administration thereof at a significantly higher concentration when compared with known combinations containing OCIF and polysaccharides, such as those disclosed in WO-A-2000/24416. Furthermore, the compositions have significantly enhanced activity in inhibiting bone resorption as compared to either the OCIF complex or the substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins alone. Indeed, the OCIF complex and the substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins exhibit a synergistic effect. The compositions and agents of the present invention are useful for preventing or treating various bone metabolic diseases such as osteoporosis, hypercalcemia, bone lytic metastasis, bone loss due to rheumatoid arthritis, osteopenia due to steroid medication, multiple myeloma, osteopenia or hypercalcemia due to renal dysfunction, renal osteodystrophy, osteoarthritis and the like.

Claims

1. A pharmaceutical composition containing:
 - (i) a complex comprising at least one substance selected from the group consisting of osteoclastogenesis inhibitory factor (OCIF), analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof; and
 - (ii) a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins.
2. A pharmaceutical composition according to claim 1, further comprising a pharmaceutically acceptable diluent or carrier.
3. A pharmaceutical composition according to claim 1 or claim 2, wherein the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said substance selected from the group consisting of polysaccharides and derivatives thereof in said complex is from 1:1 to 1:10.
4. A pharmaceutical composition according to claim 3, wherein said molecular ratio is from 1:1 to 1:8.
5. A pharmaceutical composition according to claim 3, wherein said molecular ratio is from 1:1 to 1:6.
6. A pharmaceutical composition according to any one of claims 1 to 5, wherein said substance selected from the group consisting of OCIF, analogues thereof and variants thereof is a monomer or a dimer.
7. A pharmaceutical composition according to claim 6, wherein said substance selected from the group consisting of OCIF, analogues thereof and variants thereof is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions.

8. A pharmaceutical composition according to any one of claims 1 to 7, wherein said OCIF comprises amino acids -21 to +380 of SEQ. ID. NO.1 of the sequence listing.
9. A pharmaceutical composition according to any one of claims 1 to 7, wherein said OCIF comprises amino acids +1 to +380 of SEQ. ID. NO.1 of the sequence listing.
10. A pharmaceutical composition according to any one of claims 1 to 9, wherein said polysaccharides and derivatives thereof are selected from the group consisting of hyaluronic acid, chondroitin sulfuric acid, dermatan acid, heparan acid, keratan acid, carrageenan, pectin, heparin, dextran and derivatives thereof.
11. A pharmaceutical composition according to claim 10, wherein said polysaccharide derivative is selected from dextran sulfate and salts thereof.
12. A pharmaceutical composition according to claim 11, wherein said polysaccharide derivative is a sodium salt of dextran sulfate.
13. A pharmaceutical composition according to claim 12, wherein the average molecular weight of said dextran sulfate is from 1800 to 6000.
14. A pharmaceutical composition according to any one of claims 1 to 13, wherein the strength of adsorption of said complex to heparin is lower than the strength of adsorption of the free, non-complexed OCIF or analogue or variant thereof.
15. A pharmaceutical composition according to claim 14, wherein the degree of adsorption of said complex to heparin, calculated according to the following procedure, is less than 0.7:
 - (a) a column packed with cross-linked agarose beads on which has been immobilized heparin is equilibrated with a low ionic strength buffer containing 0.1 to 0.8 M sodium chloride;
 - (b) the complex that is being tested is dissolved in the same low ionic strength buffer as used in (a) and applied to the column and a first eluate is then collected (fraction A);
 - (c) the column is then washed further with the same low ionic strength buffer as used in step (a) and a second eluate is collected (fraction B).

- (d) the column is then washed with a buffer having a high ionic strength containing 1.0 to 2.0 M sodium chloride and a third eluate is then collected (fraction C);
- (e) the amount of the complex present in each of fractions A, B and C [designated (a), (β) and (γ) respectively] is determined by an immunoassay; and
- (f) the degree of adsorption of the complex to heparin is then determined according to the following formula:

$$\text{degree of adsorption} = \frac{(\gamma)}{(\alpha) + (\beta) + (\gamma)}$$

16. A pharmaceutical composition according to any one of claims 1 to 15 comprising a complex of an OCIF or an analogue or variant thereof and dextran sulfate or a salt thereof, wherein the ratio of the number of molecules of said OCIF or analogue or variant thereof present in said complex as determined by enzyme-linked immunosorbent assay (ELISA) using anti-human OCIF monoclonal antibody OI-19 purified from a culture of a hybridoma producing antibody OI-19 (FERM BP-6420) as the antibody bound to the solid phase and anti-human OCIF monoclonal antibody OI-4 purified from a culture of a hybridoma producing antibody OI-4 (FERM BP-6419) labeled with peroxidase in the mobile phase to the number of molecules of OCIF or analogue or variant thereof present in said complex as determined by measuring the total protein content using Lowry's method is from 0.5 to 1.2.

17. A pharmaceutical composition according to any one of claims 1 to 16, wherein said complex comprising at least one substance selected from the group consisting of OCIF, analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof is produced by:

- (a) incubating said at least one substance selected from the group consisting of OCIF, analogues thereof and variants thereof and said least one substance selected from the group consisting of polysaccharides and derivatives thereof under alkaline conditions; and
- (b) then removing any free polysaccharides or derivatives thereof not bound to said OCIF, analogues thereof or variants thereof.

18. A pharmaceutical composition according to claim 17, wherein the incubation step (a) is performed at a pH of from 9.5 to 12.

19. A pharmaceutical composition according to claim 17, wherein the incubation step (a)

is performed at a pH of from 10 to 11.

20. A pharmaceutical composition according to any one of claims 17 to 19, wherein the removal of free polysaccharides or derivatives thereof in step (b) is effected by gel filtration.

21. A pharmaceutical composition according to any one of claims 1 to 20, wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is selected from non-steroidal anti-inflammatory drugs (NSAIDs), steroids and prostaglandin receptor antagonists.

22. A pharmaceutical composition according to claim 21, wherein said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID).

23. A pharmaceutical composition according to claim 22, wherein said non-steroidal anti-inflammatory drug (NSAID) is a cyclooxygenase inhibitor.

24. A pharmaceutical composition according to claim 22, wherein said non-steroidal anti-inflammatory drug (NSAID) is selected from acidic non-steroidal drugs and basic non-steroidal drugs.

25. A pharmaceutical composition according to claim 22, wherein said non-steroidal anti-inflammatory drug (NSAID) is selected from loxoprofen sodium and celecoxib.

26. A pharmaceutical composition according to any one of claims 1 to 25 wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is present in a dosage sufficient when administered alone to treat or prevent an inflammatory disease in the patient to be treated.

27. A pharmaceutical composition according to any one of claims 1 to 26 wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is present in a dosage that is not sufficient when administered alone to treat or prevent a bone metabolic disease in the patient to be treated.

28. A pharmaceutical composition according to any one of claims 1 to 26 wherein the effective dosage of said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is lower than that when said substance is used alone to treat or prevent a bone metabolic disease.

29. A pharmaceutical composition according to claim 1, wherein:

(i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions, and a polysaccharide or derivative thereof selected from the group consisting of hyaluronic acid, chondroitin sulfuric acid, dermatan acid, heparan acid, keratan acid, carrageenan, pectin, heparin, dextran and derivatives thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said substance selected from the group consisting of polysaccharides and derivatives thereof being from 1:1 to 1:8; and

(ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID).

30. A pharmaceutical composition according to claim 1, wherein:

(i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions, and a polysaccharide or derivative thereof selected from dextran sulfate and salts thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said dextran sulfate or salt thereof being from 1:1 to 1:8; and

(ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID) that is a cyclooxygenase inhibitor.

31. A pharmaceutical composition according to claim 1, wherein:

- (i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric or dimeric OCIF in which said monomer or one of the units of said OCIF dimer comprises amino acids +1 to +380 of SEQ. ID. NO.1 of the sequence listing and said polysaccharide derivative is a sodium salt of dextran sulfate having an average molecular weight of from 1500 to 12000, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate being from 1:1 to 1:8; and
- (ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID) selected from loxoprofen sodium and celecoxib.

32. A pharmaceutical composition according to claim 31, wherein the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate is from 1:1 to 1:6.

33. A pharmaceutical composition according to claim 31 or claim 32, wherein said polysaccharide derivative is a sodium salt of dextran sulfate having an average molecular weight of from 1800 to 6000.

34. A pharmaceutical composition according to any one of claims 29 to 33, wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is present in a dosage sufficient when administered alone to treat or prevent an inflammatory disease in the patient to be treated.

35. A pharmaceutical composition according to any one of claims 29 to 34, wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is present in a dosage that is not sufficient when administered alone to treat or prevent a bone metabolic disease in the patient to be treated.

36. A pharmaceutical composition according to any one of claims 29 to 34, wherein the effective dosage of said substance that suppresses the production of prostaglandins and/or that

competes with a biological action of prostaglandins is lower than that when said substance is used alone to treat or prevent a bone metabolic disease.

37. An agent for the prevention or treatment of bone metabolic diseases containing:

- (i) a complex comprising at least one substance selected from the group consisting of osteoclastogenesis inhibitory factor (OCIF), analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof; and
- (ii) a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins.

38. An agent for the prevention or treatment of bone metabolic diseases according to claim 37 wherein said complex is as defined in any one of claims 3 to 20.

39. An agent for the prevention or treatment of bone metabolic diseases according to claim 37 or claim 38, wherein said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is as defined in any one of claims 23 to 28.

40. An agent for the prevention or treatment of bone metabolic diseases according to claim 37, wherein:

- (i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions, and a polysaccharide or derivative thereof selected from the group consisting of hyaluronic acid, chondroitin sulfuric acid, dermatan acid, heparan acid, keratan acid, carrageenan, pectin, heparin, dextran and derivatives thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said substance selected from the group consisting of polysaccharides and derivatives thereof being from 1:1 to 1:8; and
- (ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID).

41. An agent for the prevention or treatment of bone metabolic diseases according to claim 37, wherein:

- (i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions, and a polysaccharide or derivative thereof selected from dextran sulfate and salts thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said dextran sulfate or salt thereof being from 1:1 to 1:8; and
- (ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID) that is a cyclooxygenase inhibitor.

42. An agent for the prevention or treatment of bone metabolic diseases according to claim 37, wherein:

- (i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric or dimeric OCIF in which said monomer or one of the units of said OCIF dimer comprises amino acids +1 to +380 of SEQ. ID. NO.1 of the sequence listing and said polysaccharide derivative is a sodium salt of dextran sulfate having an average molecular weight of from 1500 to 12000, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate being from 1:1 to 1:8; and
- (ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID) selected from loxoprofen sodium and celecoxib.

43. An agent for the prevention or treatment of bone metabolic diseases according to claim 42, wherein the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate is from 1:1 to 1:6.

44. An agent for the prevention or treatment of bone metabolic diseases according to claim 42 or claim 43, wherein said polysaccharide derivative is a sodium salt of dextran sulfate having an average molecular weight of from 1800 to 6000.

45. An agent for the prevention or treatment of bone metabolic diseases according to any one of claims 40 to 44, wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is present in a dosage sufficient when administered alone to treat or prevent an inflammatory disease in the patient to be treated.

46. An agent for the prevention or treatment of bone metabolic diseases according to any one of claims 40 to 45, wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is present in a dosage that is not sufficient when administered alone to treat or prevent a bone metabolic disease in the patient to be treated.

47. An agent for the prevention or treatment of bone metabolic diseases according to any one of claims 40 to 45, wherein the effective dosage of said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is lower than that when said substance is used alone to treat or prevent a bone metabolic disease.

48. An agent according to any one of claims 37 to 47, wherein said bone metabolic disease is selected from the group consisting of osteoporosis, osteopenia, Paget's disease, osteomyelitis, infectious focus due to loss of bone, hypercalcemia, osteoclasts, joint destruction or osteopenia due to rheumatism, osteoarthritis, loss of periodontal bone, cancer metastasis of bone, osteonecrosis or osteocyte death accompanying traumatic injury, Gaucher's disease, sickle cell anemia, lupus erythematosus systemic or nontraumatic injury, osteodystrophy, and cachexia due to solid carcinoma or cancer metastasis of bone or hematology-malignant disease.

49. A kit including a plurality of containers in which at least one of said containers contains a complex comprising at least one substance selected from the group consisting of

osteoclastogenesis inhibitory factor (OCIF), analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof, and at least one different container contains a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins.

50. A kit according to claim 49, wherein said complex is as defined in any one of claims 3 to 20.

51. A kit according to claim 49 or claim 50, wherein said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is as defined in any one of claims 23 to 28.

52. A kit according to claim 49, wherein:

(i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions, and a polysaccharide or derivative thereof selected from the group consisting of hyaluronic acid, chondroitin sulfuric acid, dermatan acid, heparan acid, keratan acid, carrageenan, pectin, heparin, dextran and derivatives thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said substance selected from the group consisting of polysaccharides and derivatives thereof being from 1:1 to 1:8; and

(ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID).

53. A kit according to claim 49, wherein:

(i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions, and a polysaccharide or derivative thereof selected from dextran

sulfate and salts thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said dextran sulfate or salt thereof being from 1:1 to 1:8; and

(ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID) that is a cyclooxygenase inhibitor.

54. A kit according to claim 49, wherein:

(i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric or dimeric OCIF in which said monomer or one of the units of said OCIF dimer comprises amino acids +1 to +380 of SEQ. ID. NO.1 of the sequence listing and said polysaccharide derivative is a sodium salt of dextran sulfate having an average molecular weight of from 1500 to 12000, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate being from 1:1 to 1:8; and

(ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID) selected from loxoprofen sodium and celecoxib.

55. A kit according to claim 54, wherein the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate is from 1:1 to 1:6.

56. A kit according to claim 54 or claim 55, wherein said polysaccharide derivative is a sodium salt of dextran sulfate having an average molecular weight of from 1800 to 6000.

57. A kit according to any one of claims 49 to 56, wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is present in a dosage sufficient when administered alone to treat or prevent an inflammatory disease in the patient to be treated.

58. A kit according to any one of claims 49 to 57, wherein said substance that suppresses

the production of prostaglandins and/or that competes with a biological action of prostaglandins is present in a dosage that is not sufficient when administered alone to treat or prevent a bone metabolic disease in the patient to be treated.

59. A kit according to any one of claims 49 to 57, wherein the effective dosage of said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is lower than that when said substance is used alone to treat or prevent a bone metabolic disease.

60. A kit according to any one of claims 49 to 59, for use in the treatment of a bone metabolic disease selected from the group consisting of osteoporosis, osteopenia, Paget's disease, osteomyelitis, infectious focus due to loss of bone, hypercalcemia, osteoclastosis, joint destruction or osteopenia due to rheumatism, osteoarthritis, loss of periodontal bone, cancer metastasis of bone, osteonecrosis or osteocyte death accompanying traumatic injury, Gaucher's disease, sickle cell anemia, lupus erythematosus systemic or nontraumatic injury, osteodystrophy, and cachexia due to solid carcinoma or cancer metastasis of bone or hematology-malignant disease.

61. A method for the prevention or treatment of bone metabolic diseases in a mammal, which may be human, comprising administering to a patient in need thereof effective amounts of the following active ingredients:

- (i) a complex comprising at least one substance selected from the group consisting of osteoclastogenesis inhibitory factor (OCIF), analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof; and
- (ii) a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins.

62. A method according to claim 61, wherein said complex is as defined in any one of claims 3 to 20.

63. A method according to claim 61 or claim 62, wherein said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is as defined in any one of claims 23 to 28.

64. A method according to claim 61, wherein:

- (i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions, and a polysaccharide or derivative thereof selected from the group consisting of hyaluronic acid, chondroitin sulfuric acid, dermatan acid, heparan acid, keratan acid, carrageenan, pectin, heparin, dextran and derivatives thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said substance selected from the group consisting of polysaccharides and derivatives thereof being from 1:1 to 1:8; and
- (ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID).

65. A method according to claim 61, wherein:

- (i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions, and a polysaccharide or derivative thereof selected from dextran sulfate and salts thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said dextran sulfate or salt thereof being from 1:1 to 1:8; and
- (ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID) that is a cyclooxygenase inhibitor.

66. A method according to claim 61, wherein:

- (i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric or dimeric OCIF in which

said monomer or one of the units of said OCIF dimer comprises amino acids +1 to +380 of SEQ. ID. NO.1 of the sequence listing and said polysaccharide derivative is a sodium salt of dextran sulfate having an average molecular weight of from 1500 to 12000, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate being from 1:1 to 1:8; and

(ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID) selected from loxoprofen sodium and celecoxib.

67. A method according to claim 66, wherein the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate is from 1:1 to 1:6.

68. A method according to claim 66 or claim 67, wherein said polysaccharide derivative is a sodium salt of dextran sulfate having an average molecular weight of from 1800 to 6000.

69. A method according to any one of claims 61 to 68, wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is administered in a dosage sufficient to treat or prevent an inflammatory disease in the patient to be treated.

70. A method according to any one of claims 61 to 69, wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is administered in a dosage that is not sufficient alone to treat or prevent a bone metabolic disease in the patient to be treated.

71. A method according to any one of claims 61 to 69, wherein the effective dosage of said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is lower than that when said substance is used alone to treat or prevent a bone metabolic disease.

72. A method according to any one of claims 61 to 71, wherein said bone metabolic disease is selected from the group consisting of osteoporosis, osteopenia, Paget's disease, osteomyelitis, infectious focus due to loss of bone, hypercalcemia, osteoclasts, joint

destruction or osteopenia due to rheumatism, osteoarthritis, loss of periodontal bone, cancer metastasis of bone, osteonecrosis or osteocyte death accompanying traumatic injury, Gaucher's disease, sickle cell anemia, lupus erythematosus systemic or nontraumatic injury, osteodystrophy, and cachexia due to solid carcinoma or cancer metastasis of bone or hemology-malignant disease.

73. Use of a complex comprising at least one substance selected from the group consisting of osteoclastogenesis inhibitory factor (OCIF), analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives thereof in the manufacture of a medicament for use with a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins for the prevention or treatment of bone metabolic diseases.

74. Use according to claim 73, wherein said complex is as defined in any one of claims 3 to 20.

75. Use according to claim 73 or claim 74, wherein said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is as defined in any one of claims 23 to 28.

76. Use according to claim 73, wherein:

(i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions, and a polysaccharide or derivative thereof selected from the group consisting of hyaluronic acid, chondroitin sulfuric acid, dermatan acid, heparan acid, keratan acid, carrageenan, pectin, heparin, dextran and derivatives thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said substance selected from the group consisting of polysaccharides and derivatives thereof being from 1:1 to 1:8; and

(ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug

(NSAID).

77. Use according to claim 73, wherein:

- (i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions, and a polysaccharide or derivative thereof selected from dextran sulfate and salts thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said dextran sulfate or salt thereof being from 1:1 to 1:8; and
- (ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID) that is a cyclooxygenase inhibitor.

78. Use according to claim 73, wherein:

- (i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric or dimeric OCIF in which said monomer or one of the units of said OCIF dimer comprises amino acids +1 to +380 of SEQ. ID. NO.1 of the sequence listing and said polysaccharide derivative is a sodium salt of dextran sulfate having an average molecular weight of from 1500 to 12000, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate being from 1:1 to 1:8; and
- (ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID) selected from loxoprofen sodium and celecoxib.

79. Use according to claim 78, wherein the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate is from 1:1 to 1:6.

80. Use according to claim 78 or claim 79, wherein said polysaccharide derivative is a

sodium salt of dextran sulfate having an average molecular weight of from 1800 to 6000.

81. Use according to any one of claims 73 to 80, wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is present in a dosage sufficient when administered alone to treat or prevent an inflammatory disease in the patient to be treated.

82. Use according to any one of claims 73 to 81, wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is present in a dosage that is not sufficient when administered alone to treat or prevent a bone metabolic disease in the patient to be treated.

83. Use according to any one of claims 73 to 81, wherein the effective dosage of said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is lower than that when said substance is used alone to treat or prevent a bone metabolic disease.

84. Use according to any one of claims 73 to 83, wherein said bone metabolic disease is selected from the group consisting of osteoporosis, osteopenia, Paget's disease, osteomyelitis, infectious focus due to loss of bone, hypercalcemia, osteoclastosis, joint destruction or osteopenia due to rheumatism, osteoarthritis, loss of periodontal bone, cancer metastasis of bone, osteonecrosis or osteocyte death accompanying traumatic injury, Gaucher's disease, sickle cell anemia, lupus erythematosus systemic or nontraumatic injury, osteodystrophy, and cachexia due to solid carcinoma or cancer metastasis of bone or hemology-malignant disease.

85. Use of a substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins in the manufacture of a medicament for use with a complex comprising at least one substance selected from the group consisting of osteoclastogenesis inhibitory factor (OCIF), analogues thereof and variants thereof, which is bound to at least one substance selected from the group consisting of polysaccharides and derivatives for the prevention or treatment of bone metabolic diseases.

86. Use according to claim 85, wherein said complex is as defined in any one of claims 3 to 20.

87. Use according to claim 85 or claim 86, wherein said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is as defined in any one of claims 23 to 28.

88. Use according to claim 85, wherein:

(i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions, and a polysaccharide or derivative thereof selected from the group consisting of hyaluronic acid, chondroitin sulfuric acid, dermatan acid, heparan acid, keratan acid, carrageenan, pectin, heparin, dextran and derivatives thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said substance selected from the group consisting of polysaccharides and derivatives thereof being from 1:1 to 1:8; and

(ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID).

89. Use according to claim 85, wherein:

(i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric OCIF having a molecular weight as measured by SDS-PAGE under non-reducing conditions of about 60000 or human dimeric OCIF having a molecular weight of about 120000 as measured by SDS-PAGE under non-reducing conditions, and a polysaccharide or derivative thereof selected from dextran sulfate and salts thereof, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said dextran sulfate or salt thereof being from 1:1 to 1:8; and

(ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID) that is a cyclooxygenase inhibitor.

90. Use according to claim 85, wherein:

- (i) said complex comprises a substance selected from the group consisting of OCIF, analogues thereof and variants thereof which is human monomeric or dimeric OCIF in which said monomer or one of the units of said OCIF dimer comprises amino acids +1 to +380 of SEQ. ID. NO.1 of the sequence listing and said polysaccharide derivative is a sodium salt of dextran sulfate having an average molecular weight of from 1500 to 12000, the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate being from 1:1 to 1:8; and
- (ii) said substance that suppresses the production of prostaglandins and/or a substance that competes with a biological action of prostaglandins is a non-steroidal anti-inflammatory drug (NSAID) selected from loxoprofen sodium and celecoxib.

91. Use according to claim 90, wherein the molecular ratio of said substance selected from the group consisting of OCIF, analogues thereof and variants thereof to said sodium salt of dextran sulfate being from 1:1 to 1:6.

92. Use according to claim 90 or claim 91, wherein said polysaccharide derivative is a sodium salt of dextran sulfate having an average molecular weight of from 1800 to 6000.

93. Use according to any one of claims 85 to 92, wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is present in a dosage sufficient when administered alone to treat or prevent an inflammatory disease in the patient to be treated.

94. Use according to any one of claims 85 to 93, wherein said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is present in a dosage that is not sufficient when administered alone to treat or prevent a bone metabolic disease in the patient to be treated.

95. Use according to any one of claims 85 to 93, wherein the effective dosage of said substance that suppresses the production of prostaglandins and/or that competes with a biological action of prostaglandins is lower than that when said substance is used alone to treat or prevent a bone metabolic disease.

96. Use according to any one of claims 85 to 95, wherein said bone metabolic disease is selected from the group consisting of osteoporosis, osteopenia, Paget's disease, osteomyelitis, infectious focus due to loss of bone, hypercalcemia, osteoclasia, joint destruction or osteopenia due to rheumatism, osteoarthritis, loss of periodontal bone, cancer metastasis of bone, osteonecrosis or osteocyte death accompanying traumatic injury, Gaucher's disease, sickle cell anemia, lupus erythematosus systemic or nontraumatic injury, osteodystrophy, and cachexia due to solid carcinoma or cancer metastasis of bone or hematology-malignant disease.

SEQUENCE LISTING

<110> Sankyo Company, Limited

<120> A Pharmaceutical Composition

<130> FP-0303WO

<150> JP 2002-055356

<151> 2002-03-01

<160> 1

<170> PatentIn version 3.1

<210> 1

<211> 401

<212> PRT

<213> Homo sapiens

<220>

<221> SIGNAL

<222> (-21)..(-1)

<223>

<220>

<221> mat_peptide

<222> (+1)..(+380)

<223>

<400> 1

Met Asn Asn Leu Leu Cys Cys Ala Leu Val Phe Leu Asp Ile Ser Ile
-20 -15 -10

2/3

Lys Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp
 -5 -1 1 5 10

Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr
 15 20 25

Tyr Leu Lys Gln His Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro
 30 35 40

Cys Pro Asp His Tyr Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
 45 50 55

Leu Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu
 60 65 70 75

Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr
 80 85 90

Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe
 95 100 105

Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg
 110 115 120

Cys Pro Asp Gly Phe Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys
 125 130 135

Arg Lys His Thr Asn Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys
 140 145 150 155

Gly Asn Ala Thr His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr
 160 165 170

Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg
 175 180 185

Phe Ala Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val
 190 195 200

Asp Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile
 205 210 215

Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu
 220 225 230 235

Trp Lys His Gln Asn Lys Asp Gln Asp Ile Val Lys Lys Ile Ile Gln
 240 245 250

Asp Ile Asp Leu Cys Glu Asn Ser Val Gln Arg His Ile Gly His Ala
 255 260 265

3/3

Asn Leu Thr Phe Glu Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly
270 275 280

Lys Lys Val Gly Ala Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys
285 290 295

Pro Ser Asp Gln Ile Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn
300 305 310 315

Gly Asp Gln Asp Thr Leu Lys Gly Leu Met His Ala Leu Lys His Ser
320 325 330

Lys Thr Tyr His Phe Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr
335 340 345

Ile Arg Phe Leu His Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu
350 355 360

Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys
365 370 375

Leu
380

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K45/06 A61K38/17 A61P19/08 A61P19/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, EMBASE, BIOSIS, CHEM ABS Data, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 1 127 578 A (SNOW BRAND MILK PROD CO LTD ;SANKYO CO (JP)) 29 August 2001 (2001-08-29) cited in the application paragraph '0005! paragraph '0016! paragraph '0019!; examples 2-8 ---	1-96
P,A	EP 1 270 015 A (SANKYO CO) 2 January 2003 (2003-01-02) cited in the application paragraph '0012! paragraph '0014! table 8 --- -/--	1-96

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the International filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the International filing date but later than the priority date claimed

T later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

* & * document member of the same patent family

Date of the actual completion of the International search

14 May 2003

Date of mailing of the International search report

02/06/2003

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Pilling, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 01 44472 A (AMGEN INC ; BENNETT BRIAN D (US); FOX GARY M (US); JING SHUQIAN (US) 21 June 2001 (2001-06-21) page 95, line 12 -page 96, line 7 page 96, line 27 - line 28 page 100, line 29 - line 34 page 101, line 16 page 101, line 27 -page 105, line 24 -----	1-96
A	WO 01 03719 A (AMGEN INC) 18 January 2001 (2001-01-18) cited in the application page 4, line 30 -page 5, line 3 page 71, line 18 -page 72, line 12 page 72, line 27 - line 28 -----	1-96

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 1127578	A	29-08-2001	AU 755422 B2	12-12-2002
			AU 6487799 A	15-05-2000
			BR 9914834 A	14-08-2001
			CA 2347107 A1	04-05-2000
			EP 1127578 A1	29-08-2001
			HU 0104126 A2	28-03-2002
			NO 20012106 A	21-06-2001
			US 2001031725 A1	18-10-2001
			CN 1325310 T	05-12-2001
			CZ 20011378 A3	17-10-2001
			WO 0024416 A1	04-05-2000
			PL 347559 A1	08-04-2002
			TR 200101146 T2	21-09-2001
EP 1270015	A	02-01-2003	CA 2392383 A1	29-12-2002
			EP 1270015 A2	02-01-2003
			NO 20023144 A	30-12-2002
			PL 354799 A1	30-12-2002
			US 2003045456 A1	06-03-2003
WO 0144472	A	21-06-2001	AU 2262501 A	25-06-2001
			CA 2394536 A1	21-06-2001
			EP 1238077 A1	11-09-2002
			WO 0144472 A1	21-06-2001
WO 0103719	A	18-01-2001	AU 6078500 A	30-01-2001
			WO 0103719 A2	18-01-2001

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☒ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☐ **FADED TEXT OR DRAWING**

☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.